A STUDY OF PREVALANCE OF VANCOMYCIN RESISTANCE AMONG THE ENTEROCOCCUS SPP. ISOLATED FROM VARIOUS CLINICAL SMAPLES IN A TERTIARY CARE HOSPITAL IN CHENNAI

Dissertation Submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY

in partial fulfillment of the regulations

for the award of the degree of

M.D. (MICROBIOLOGY)

BRANCH – IV

GOVT. STANLEY MEDICAL COLLEGE & HOSPITAL

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY

CHENNAI, INDIA.

APRIL 2013

CERTIFICATE

This is to certify that this dissertation entitled **"A STUDY OF PREVALANCE OF VANCOMYCIN RESISTANCE AMONG THE ENTEROCOCCUS SPP. ISOLATED FROM VARIOUS CLINICAL SMAPLES IN A TERTIARY CARE HOSPITAL IN CHENNAI"** is the bonafide original work done by **Dr. S.NALAYINI,** Post graduate in Microbiology, under my overall supervision and guidance in the department of Microbiology, Stanley Medical College, Chennai, in partial fulfilment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Microbiology (Branch IV).**

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DECLARATION

I solemnly declare that this dissertation **"A STUDY OF PREVALANCE OF VANCOMYCIN RESISTANCE AMONG THE ENTEROCOCCUS SPP. ISOLATED FROM VARIOUS CLINICAL SMAPLES IN A TERTIARY CARE HOSPITAL IN CHENNAI "** is the bonafide work done by me at the Department of Microbiology, Government Stanley Medical College Hospital, Chennai, under the guidance and supervision of **Prof. Dr.R.SELVI, M.D.,** Professor and head of department of Microbiology, Government Stanley Medical College, Chennai-600 001.

This dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the University regulations for the award of degree of M.D. Branch IV Microbiology examinations to be held in April 2013.

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ACKNOWLEDGEMENT

I offer my sincere thanks to the **DEAN, Dr.S.GEETHALAKSHMI, M.D,Ph.D** Government Stanley Medical College & Hospital,for giving me permission to do this dissertation work and use the resources of this institution. I express my gratitude to **DR, R SELVI, M.D,** Professor & Head, for the invaluable guidance , support and help.

I express my sincere thanks to our Assistant Professors Dr. VASUMATHI,M.D, Dr.EUNICE SWARNA JACOB,M.D, Dr.DAVID AGATHA,M.D, Dr.HEMALATHA,M.D, Dr.B.SHANTHI,M.D, Dr. PRABAKARAN, M.D, Dr. SUBHA,M.D, Dr.PAVENDAN,M.D, and Dr.USHA KRISHNAN for their invaluable support, advice and timely help in shaping and completion of this project work.

I offer my special thanks to my friend Dr. J. Priyadharisini , my batchmates Dr. Swapna .M, Dr.E.Arthi, and my department colleagues for their kind support and help. I thank wholeheartedly my friends, my parents and my husband for the invaluable moral support, kindness they showered on me.

I thank specially to the Prof. Dr.V.Balaji , Department of Microbiology, CMC Vellore for the technical support by providing the QC control strains for this study.

I specially thank all the technical staff and other staff members of our department for their invaluable contributions which is of great help to complete this work. I also thank all those who helped in some way to bring this project work successfully.

Last but not the least , I sincerely thank all the patients -the participants of this study , the centre of this whole project.

CONTENTS

INTRODUCTION AND BACKGROUND :

The *Enterococci* are normal commensal microorganisms , considered virtually as harmless bacteria. Normally it is a part of the indigenous flora of the intestinal tract, oral cavity and genitourinary tract of humans and animals. They have long been known as` Faecal *Streptococci'* , a potential human pathogen, capable of causing a variety of infections in the community as well as in the hospital. During the past few decades , they have emerged as an important nosocomial pathogen exhibiting multiple drug resistance, contributing significantly to patient morbidity and mortality[44]. It is due to their ability to survive and disseminate in hospital environment which is mainly attributed to their intrinsic resistance to many of the commonly used antibiotics and especially to their ability to acquire resistance to most of the currently available antibiotics either by mutation or through transfer of mobile genetic elements carrying resistance genes/ virulence factors[11].

They cause serious infections like endocarditis and bacteremia, meningitis , intra abdominal and pelvic infections, burn and surgical site wound infections in both immune-competent and immune-compromised individuals. They pose a special risk in causing infection of the catheters and various other implanted medical devices in critically ill patients and

also cause late onset sepsis , pneumonia and meningitis in neonates[7]. In the 2006-2007 report from Centers for Disease Control and Prevention (CDC) *Enterococci* account for about 12% of health care associated infections (HAI) and ranks third most common multi-drug resistant pathogen causing HAI[12]. Since the intial report of emergence of Vancomycin resistant *Enterococci* (**VRE**) reported in United kingdom and France , there have been increasing incidence of VRE being reported from various other countries like Australia, Canada, Germany, Malaysia, Spain and United States [11].

Because of their intrinsic and acquired resistance to the commonly used broad spectrum antibiotics, clinician are left with fewer options for treatment of VRE infections especially in debilitated and seriously ill patients. The alarming thing is the increasing evidence of potential risk of transfer of Vancomycin resistance gene from VRE to various Gram-positive microorganisms especially *Staphylococcus aureus* through conjugative plasmids, which worsens the scenario further[11]. Hence it is an essential part of the surveillance system of each and every hospital setup to monitor continuously such VRE infections and to assess the antibiotic susceptibility pattern of VRE isolates. Assessment of the prevalence and changing trends of VRE infections are of immense help

in planning infection control measures which should be implemented in the hospital and also in the community to reduce the mortality and morbidity caused by these VRE infections. In view of the above perspective, the present study is carried out in our tertiary care hospital in Chennai, South India to assess the prevalence and the antibiotic susceptibility pattern of Vancomycin Resistant *Enterococci* in our region.

AIMS:

To study the prevalence of vancomycin resistance among the *Enterococcal* species isolated from various clinical samples such as urine, blood, pus, tissue fluids and feces obtained from the patients of a tertiary care hospital.

OBJECTIVES:

1. Isolation and identification of *Enterococci* from various clinical samples by standard techniques.
2. Characterization of the *Enterococcal* isolates to the species level.
3. Assessment of the Antibiotic susceptibility pattern of these isolates by standard techniques.
4. To study the specific resistance mechanisms like resistance to penicillins, High level Aminoglycoside resistance (HLAR) by standard techniques.
5. To study the vancomycin resistance among the *Enterococcal* isolates and its characterization by standard phenotypic methods.
6. To assess the prevalence of VanA genotype among the Vancomycin Resistant *Enterococci* by Polymease Chain Reaction (PCR)assay for VanA gene.

REVIEW OF LITERATURE:

DESCRIPTION OF GENUS:

The *Enterococci* were mentioned first in French literature in 1899 as "enterococque" and isolated from a case of endocarditis in the same year[6,7]. It was initially designated as "*Micrococcus zymogenes"* and from 1906 onwards as "*Streptococcus faecalis"* and later in 1984 , assigned a separate genus "*Enterococcus"*based on DNA hybridization studies. Around 18 species have been identified from clinical isolates, the predominant one being *E.faecalis(* 80 to 90%) and *E.faecium* (5 to10%)[7,11] . Other species like *E.gallinarum.E.durans,E.hirae,E.avium* are less frequently isolated[7].

- *Enterococci* are gram-positive cocci , oval in shape, arranged as single cell, pairs ,short chains and rarely even long chains.
- They are non-motile (except *E. gallinarum, E.casseliflavus*) and non capsulated[16].
- They are facultative anaerobes producing lactic acid but gas is not produced.
- They are generally catalase negative (few strains of *E.faecalis* produce weak effervescence when grown on blood agar) and are usually α- hemolytic or non-hemolytic. Some cultures of

E.faecalis are β-hemolytic on human, horse and rabbit blood agar but non-hemolytic on sheep blood.

- About 80% of *Enterococci* react with Lancefield group D antigen[28].
- They are able to grow at temperature between 10ºC and 45ºC, showing optimum growth at35 to 37ºC.
- On blood agar after 24 hrs growth, they produce white to grey coloured colonies, 1to 2mm diameter and on MacConkey agar 0.5- 1mm majenta pink coloured colonies are produced[16].
- Majority of them grow in broth containing 6.5% NaCl, at pH 9.6, and survive heating at 60ºC for 30 minutes [11,37] .
- They hydrolyse esculin in the presence of 40% bile (Bile esculin test).
- They hydrolyse L-pyrrolidonyl –β-naphthylamide (PYR) to produce pyrolidonyl arylamidase and also hydrolyse leucine –β-naphthylamide producing leucine aminopeptidase (LAP).
- Some species produce pigment(yellowish) (*E.casseliflavus, Emundtii, E.pallens, E.sulfureus and E. gilvus*).
- *Enterococci* don't produce cytochrome oxidase enzymes[45]

- Most of the *Enteococcial* species except a few, react with Accuprobe *Enterococcus* genetic probe and this can be used to confirm an unknown strain as *Enterococcus.*

GENOME:

The genome size is about 2 to 3.5 Mb and the G+ C content is 32 to 44 mol %. The genome of *E.faecalis* V583, the first vancomycin resistant clinical isolate from United States is completely sequenced and is useful in various research purposes [45]. The genome of > 80 *enterococcal* strains has been sequenced [7]. The genetic diversity of enterococci is due the acquisition of mobile DNA like plasmids, transposons and phages and also a result of recombination of "core" genomes. The medically important *E.faecium* harbors an accessory genome into which exogenous genetic elements like Phage DNA are incorporated[7] .It also harbors pathogenicity island[11,21], which is a large genetic element carrying virulence associated genes and plasmids with antibiotic resistant determinants.

EPIDEMIOLOGY:

Enterococci are distributed widely in nature as they can be found in soil, plants, water, food, animals, birds and reptiles. In humans, they are predominant commensals of gastrointestinal and biliary tract and

found in less numbers in genitourinary tract, oral cavity and perineal skin[28,45]. The prevalence of different *Enterococcal* species is influenced by host factors, age, diet, underlying disease and prior antimicrobial therapy. The most abundant species colonizing the gastrointestinal tract is *E.faecalis* and others like *E.faecium, E.casseliflavus, E.durans, E.gallinarum* in variable proportions [7,45]. The gastrointestinal tract represents the endogenous source/reservoir for disease causing strains , they may migrate from here to infection site and also disseminate to other hosts and to the environment [45]. They are used as indicators of hygienic quality and fecal contamination of food, milk and drinking water[45].

The predominant species encountered in nosocomial infections is *E.faecalis,* however *E.faecium* is on the rise in the past few decades. Now-a-days *E.faecium* is being isolated as common as *E.faecalis* in hospital associated infections[7]. About 30% of enterococcal infections are caused by VRE, with *E.faecium* being the commonest isolate (>90%) [6].

PATHOGENESIS:

Since *Enterococci* are opportunistic pathogens , these infections may arise from translocation of *Enterococcal* cells from the site of

colonization of the gastrointestinal system to other sites in host and to the hospital environment[6]. However the pathogenesis of *Enterococcal* infections is only poorly understood. The source could be either endogenous or exogenous , however studies show that there may exist a subset of virulent lineage with greater ability to cause disease and often responsible for outbreaks and infections of epidemic proportions.

Acquisition of new traits (pathogenecity island, virulence associated genes,multi-drug resistance etc..) and their intrinsic resistance to the commonly used antibiotics allow the bacterium to overcome host defense mechanisms. These traits along with the acquired antimicrobial resistance differentiates the virulent pathogenic strains from the commensals. These along with the changed dynamics of host – commensal relationships like diminished host immunity, host injury and broad spectrum antibiotic use, promote colonization of new niches favoring infections and its dissemination to other tissues and environment[45].In particular use of drugs that are excreted in bile and broad spectrum antibiotics like cephalosporins (active against anaerobes and gram negative bacteria) eradicate the competing components of intestinal flora and also cause suppression of important immunological signals(e.g lectin RegIIIγ) that keep the *Enterococi* in low numbers

normally. Thus the virulent strains exhibit survival advantage over others to persist for longer periods in the host and environment and play a prominent role as nosocomial pathogens. The important factor that *Enterococci* can transfer the resistant determinants to other Gram positive bacteria [6,11], further increases their clinical significance.

VIRULENCE FACTORS:

The potential virulence factors(*Enterococcal* secreted factors , *Enterococcal* surface components) identified in *Enterococcal* isolates and proposed to play a role in pathogenesis are as follows[6,7,11,28,45]

Enterococcal secereted factors:

- Cytolysin /hemolysin- a heterodimeric toxin secreted by some strains of *E.faecalis* lyses human, rabbit, equine and bovine erythrocytes (but not sheep rbcs) and polymorphonuclear leucocytes and macrophages, playing significant role in endocarditis and rabbit endophthalmitis models[28]
- Enterococcal proteases-The gelatinase (GelE), serine protease(SprE) of *E.faecalis* mediate virulence by several mechanisms like degradation of host tissues and modification of immune components.

- ❖ Coccolysin, an extracellular metalloendopeptidase secereted by *E.faealis* strains mediate virulence by inactivating the vasoactive peptide –endothelin.
- ❖ Extracellular superoxide, secereted in large amounts by most of *E.faecalis,E.faecium* strains enhance virulence in mixed flora abcesses.

Enterococcal surface proteins:

- ❖ Aggregation substance –a plasmid-encoded surface protein which promotes enterococcal adherence to epithelial cells and clumping of organisms facilitating plasmid exchange . It also favors growth of cardiac vegetations in rabbit endocarditis model[28,45].
- ❖ The surface proteins – Ace(adhesion of collagen of *E.faecalis*) and Acm(homologue adhesion of *E.faecium)* are Microbial surface components recognizing adhesive matrix molecules (MSCRAMM) mediate bacterial attachment to host proteins like collagen,fibrinogen , fibronectin.
- ❖ Other surface proteins similar to MSCRAMM playing role in virulence by mediating bacterial attachment are second collagen adhesion of *E.faecium*(Scm),enterococcal surface protein (Esp_{fc})

of *E.faecalis* and of *E.faecium*(Esp_{fm}), surface proteins (Fms) of *E.faecium*,SgrA(which binds to basal lamina components).EcbA(binds to collagen type V)(har,Murra).

- Lipoteichoic acid (group D antigen) functions in modulating immune response by inducing the production of TNF and Interferons(kony).
- Pili is present in both *E.faecalis* and *E.faecium* which mediates attachment and invasion into host tissues and are targets of immunotherapy.
- Bio-films produced by enterococci alter the efficacy of anti-enterococcl agents and play a role in pathogenesis of experimental endocarditis and UTI[11,44].
- Polysaccharides (on bacterial cell surface) contribute to pathogenicity by interfering with phagocytosis. *E.faecalis* possesses three distinct classes of capsular polysaccharides which are potential targets of immunotherapy.[7]

Other virulence factors:

- *E.faecalis* stress protein Gls24 – responsible for enterococcal resistance to bile salts important in pathogenicity of endocarditis .

- *hyl* $_{Efm}$- containing plamids found in *E.faecium* , increase the colonizing capacity.

CLINICAL SYNDROMES:[6,7]

Urinary tract infections:

Nosocomial UTI is the most common infection caused by the *Enterococci ,* associated with anatomic abnormalities of genitourinary tract, instrumentation, indwelling catheters, prior antibiotic use and recurrent UTIs. It is the third most common agent isolated from catheter-associated UTIs in the United States with *E.faecium* being the predominant species(40%) followed by *E.faecalis* (25%) and other species(35%)[6].In the hospital setting differentiating infection from colonization may be difficult and the factors like presence of leucocytes in urine along with systemic manifestations(fever), local symptoms and signs , and a colony count of > 10^5 CFU/ml may help in this regard.[6,7]. Removal of catheter itself may be enough to eradicate this agent. The complications associated with *Enterococcal* UTI are pyelnephritis, perinephric abcess and recurrent bacteremias[6,7].

Bacteremia and Endocarditis:

Bacteremia without endocarditis is the most common presentation of these two with *Enterococci* being the leading cause of nosocomial

bacteremias. Intravascular catheters and other devices are the commonest sources and others like genitourinary and biliary tracts, pelvic and intra abdominal foci, UTIs, wound and bone infections also contribute. *E.faecium* bloodstream infections carry the worse prognosis because of higher prevalence of ampicillin and vancomycin resistance and fewer therapeutic options. The association between *Enterococcal* bacteremia, meningitis and *Strongyloides* hyperinfection syndrome in immunocompromised individuals is well known[7,11].

Enterococci are important causative agents of both community acquired and hospital associated endocarditis especially in elderly, debilitated patients with comorbid conditions[6]. It can affect both native and prosthetic valves, mitral and aortic valves affected commonly. *E.faecalis* is isolated more frequently than *E.faecium* and other species. Malignant and inflammatory conditions and procedures involving genito urinary or gartrointestinal tracts serve as the source of origin. Typical presentation involves a subacute course with fever, malaise, weight loss , cardiac murmur and lessfrequent peripheral signs. Heart failure is the common complication followed by embolic phenomina,the most common end organ being brain. Mortality is mainly due to heart failure or embolization and the overall mortality rate ranges from 11% to 35%[6].

Meningitis:

Enterococci are uncommon agents of meningitis accounting for only 4% of all meningitis cases and presents as two forms- Spontaneous and post operative meningitis. The most common species isolated is *E.faecalis* followed by *E.faecium* , *E.gallinarum* and other species. Spontaneous meningitis is a community acquired in patients with many comorbid conditions like Diabetes, renal failure, malignancy, immunosuppression and in children with CNS pathology. Post operative meningitis is hospital acquired in the presence of shunt devices. Both forms have similar clinical features – acute course with fever, altered mental status and signs of meningial irritation. Complications include hydrocephalus, brain abcess and stroke with overall mortality of 20%[6,7].

Intraabdominal,pelvic and soft tissue infections:

Enterococci produce spontaneous peritonitis in cirrhotic patients and patients on chronic ambulatory peritoneal dialysis. It is usually isolated along with gram negatives and anaerobes and its presence indicates treatment failure and increases the postoperative complications, and mortality. The emergence and spread of VRE and multi-drug resistant *E.faecium* isolates worsens the situation further.

Enterococci rank third as the causative agents of hospital acquired surgical-site infections[6,12]. *E.faecalis* is the common species

isolated *Enterococci* often colonize the decubitus ulcer and diabetic foot and can be a source of bone infections.

Other infections:

It also causes neonanatal infections like late onset sepsis , bacteremia, pneumonia and UTI in the presence risk factors like prematurity, low birth weight and indwelling devices. It also causes bone and joint infections.

LABORATORY DIAGNOSIS:

Collection, transport and storage of specimens:

The specimens usually submitted are blood, urine, wound exudates and secretions from other sites/ swab specimens and standard method of collection of these samples is adequate [45].

As *Enterococci* are nonfastidious organisms, relatively resistant to adverse environmental conditions, no special methods of transport and storage of clinical specimens is needed. Specimens can be transported to the laboratory using any of the transport medium or on swabs that are kept dry [44].

Enrerococci grown on agar slants of Brain heart infusion agar/Tryptic soy agar can be stored for several months at 4ºC. The preferable methods would be storage as frozen cultures at - 70ºC of heavy cell suspensions made in defibrinated sheep or rabbit blood or in

10% skimmed milk solution containing 10% glycerol. They can also be stored in cryo preservative media at -20ºC for many years. Lyophilized cultures can be stored indefinitely[44].

Direct examination:

The direct microscopic examination of gram stained smears of normally sterile clinical specimens like blood is useful in diagnosing *Enterococcal* infections. However, only a presumptive report of "presence of Gram positive cocci" can be given in case of non sterile specimens. Direct detection of *Enterococci* especially VRE from clinical specimens and surveillance specimens (feces,rectal swab) by using conventional and real-time PCR based methods have been developed and evaluated . A multiplex real-time PCR assay (Light cycler septifast test) for rapid detection and identification of major pathogens of nosocomial bacteremia in whole blood is available for use in the United States.

Isolation procedures:

Clinical specimens from normally sterile body sites, can be plated onto tryptic soy agar, brain heart infusion agar or blood agar base containing either 5%sheep,horse or rabbit blood for primary isolation of *Enterococci* . Samples for blood culture are inoculated into conventional blood culture systems. Most of the clinically relevant species grow well

at 35 to 37ºC. For specimens obtained from non sterile sites especially when contaminated with gram negative bacilli, selective media containing sodium azide, bile salts, antibiotics and esculin , tetrazolium can be used for primary isolation. However not all *Enterococci* grow on selective media. Use of enrichment broth(Enterococcosel broth- BEA medium with 6µg vancomycin) increases the recovery rate of *Enterococci* especially VRE from feces and rectal samples especially surveillance specimens. Various chromogenic media from different manufacturers also have been evaluated for the primary isolation.

Identification of *Enterococcus* species:

The genus identification of a catalase negative, Gram positive coccus as "*Enterococcus"* is based on the above said tests in genus description. *Enterococal* species can be classified into five physiological groups of species as proposed by Facklam and Collins, based on acid production from mannitol and sorbose and hydrolysis of arginine[45] . Further speciation is based on acid production from sugars like arabinose, sorbitol, raffinose, sucrose, pyruvate, trehalose and reduction of 0.04% tellurite, motility and pigment production.

- Group I – consists of 9 species. They produce acid from mannitol and sorbose, arginine is not hydrolysed. *E.avium,*

E.raffinosus are the clinically relevant species in this group.Others are *E.gilvus, E.pallens, E.saccharolyticus, E.malodoratus,E.pseudoavium, E.divriesei* and *E.hawaiiensis.*

- Group II – consists of 8species. They produce acid from mannitol only and not from sorbose andarginine is hydrolysed. Majority of the isolates recovered from human sources like*E.faecalis ,E..faecium, E.gallinarum, E.casseliflavus* belong to this group. Others are *E.mundtii, E.haemoperoxidus, E.sanguinicola, E.ythailandicus.*
- Group III –consists of 6 species. They don't produce acid from mannitol and sorbose, but hydrolyse arginine. It includes *E.dispar, E.canintestini, E.hirae, E.durans, E.ratti,* and *E.villorum.*
- Group IV – includes 8 species. They don't produce acid from both mannitol and sorbose and arginine is not hydrolysed. It includes *E.caccae, E.cecorum, E.aquimarinus, Ephoeniculicola, E.sulfureus, E.asini, E.silesiacus, E.termitis.*
- Group V – consists of 6 species. They ferment only mannitol producing acid but not sorbose and arginine also

not hydrolysed. *E.canis, E.columbae, E.moraviensis, E.camelliae,E.hermanniensis,* and *E.italicus* are the species included in this group.

Various **commercial identification systems** – manual, semi-automated and automated systems like API 20S, API Rapid ID32 STREP, Crystal gram positive ID system, Gram positive identification card of Vitek system etc..are available. These are reliable for the detection of most common species *E.faecalis* and to a lesser extent *E.faecium.*

Molecular methods[45] like SDS polyacrylamide gel electrophoresis analysis of whole cell proteins (WCP) profile and 16S rRNA gene sequencing are extensively evaluated for identification of different *Enterococcal* species. The 16S rRNA gene (1500 base span) sequencing is the most frequently used molecular method for identification of *Enteococcus* species. However it should be used along with phenotypic characterization for diagnostic purposes. PCR based techniques use amplification of *ddl* or *sodA* genes for accurate and rapid identification are also available. DNA probe kit Accuprobe(Gen-Probe,Inc) directly detects *Enterococci* in blood. FISH (Fluorescent In

Situ Hybridization) techniques (PNA FISH) been evaluated for identification of *Enterococci* from positive blood cultures.

Analysis of chromosomal restriction profiles by pulsed field gel electrophoresis (PFGE) has been extensively evaluated for strain typing and epidemiological outbreaks allowing the identification of predominant clonal complexes and resistance genes - HLAR high level aminoglycosides resistance and VRE- vancomycin resistance. Among the recent methods like MLST- Multilocus sequence typing and MLVA- multi locus variable number tandem repeat analysis , MLVA is less expensive and rapid compared to MLST.

ANTIMICROBIAL SUSCEPTIBILITY AND RESISTANCE MECHANISMS:[6,7,8,11,35,45]

Enterococci exhibit both intrinsic and acquired resistance to several of the commonly used antibiotics. Intrinsic resistance is chromosomally mediated and is found in all or most of *Enterococci* and is against β-lactams (cephalosporins, penicillinase resistant penicillins), lower concentration of aminoglycosides, fluoroquinolones, trimethoprim-sulfmetoxazole and clindamycin. However the most recommended regimen of combination of a cell wall active agent (β-

lactams) and an aminoglycoside, for serious infections like endocarditis and in immunocompromised patients, overcomes the intrinsic resistance by exerting synergistic bactericidal killing. This is achieved by the facilitation of aminoglycoside entry into the bacteria by the damage caused by the cell wall active agent.

Acquired resistance is variable and results from either mutations in existing DNA or acquisition of new genetic determinants carried on plasmids / transposans. It rather confers resistance to several classes of antibiotic agents including chloramphenicol, tetracyclines, MLS-macrolides-lincosamide-streptogramins, higher concenrtrations of aminoglycosides and β-lactams, glycopeptides, rifampin and nitrofurantoin than to a single agent. Recent reports highlight the emergence of resistance to the newer agents like linezolid, daptomycin and quinupristin-dalfopristin[7].

β-LACTAM RESISTNCE:

High level resistance to penicillin and ampicillin, as a result of low affinity penicillin binding proteins (especially PBP5- amount produced proportionate to resistance) is a common occurrence among enterococci. β- lactamase production in *Enterococci* is a rarely identified one and is constitutively expressed and is of low level

resistance, whereas in other bacteria it is an inducible resistance[11] . *Enterococci* susceptible to penicillin are predictably susceptible to ampicillin and other β-lactams. But ampicillin susceptibility doesn't predict susceptibility to penicillin , separate testing with penicillin is required. Ampicillin susceptibility can be used to predict imipenem susceptibility in case of *E.faecalis* species. Nitrocefin based test is needed for the reliable detection of β-lactamase production in *Enterococci,* as disc diffusion and dilution methods are not reliable.

RESISTANCE TO AMINOGLYCOSIDES (HLAR- High Level Aminoglycoside Resistance):

Aminoglycoside resistance in *Enterococci* is due to two types of mechanisms;

a) Low permeability to aminoglycosides leading to moderate level resistance (MIC 62-500µg/ml), which can be overcome by synergism with cell wall active agents
b) Production of inactivating enzymes (acetyl transferase,adenyl transferase) or ribosomally mediated resistance which is of high level (MIC ≥ 2,000µg /ml).

Gentamicin resistance is primarily due to the presence of inactivating enzyme 2”-phosphotransferase-6’-acetyltransferase, resulting in resistance to gentamicin tobramycin , amikacin and other aminoglycosides except streptomycin. The enzyme adenyl transferase operates in streptomycin resistance strains, but these strains are susceptible to gentamicin. Hence gentamicin and streptomycin – both should be tested individually to predict the resistance to aminoglycosides.

Resistance to either aminoglycoside or cell wall active agent (β-lactams, glycopeptides) results in resistance to the synergistic killing of *Enterococci* by the combination therapy and constitute a critical therapeutic problem. Hence detection of resistance to these agents is critical for the prediction of synergy of the combination therapy. Screening of the clinical *Enterococcal* isolates to detect high level aminoglycoside resistance(HLAR) can be done by either disc diffusion using high level gentamicin(120µg)(HLG) and high level streptomycin(300µg)(HLS) discs or single concentration agar dilution method gentamicin ≥ 500µg/ml, streptomycin ≥2000µg/ml) are available. Resistance to streptomycin is due to altered ribosomal target and modifying enzymes, whereas resistance to gentamicin is due to

modifying enzymes encoded by altered genes. Gentamycin resistance predicts resistance to other aminoglycosides except streptomycin where as streptomycin resistance indicates resistance to streptomycin only.

Emergence of Vancomycin resistance in *Enterococci* worldwide poses serious problems in managing these patients as only very few options remain there for treatment. Vancomycin resistance is highly prevalent in *E.faecium* than in *E,faecalis,* the detailed discussion of which is presented in the following pages.

TREATMENT :

The suggested therapeutic options for the treatment of serious *Enterococcal* infections like endocarditis, bacteremia is combination therapy with a cell wall active agent (β-lactams,glycopeptides) and an aminoglycoside. Among the β-lactams, aminopenicillins (ampicillin, amoxicillin) and ureidopenicillins (piperacillin) are the most active ones followed by penicillinG and imipenem. The only two aminoglycosides recommended for synergistic therapy are gentamicin and streptomycin. Aminoglycoside monotherapy is not effective. Vancomycin is an alternative to β-lactams for *E.faecalis* infections but less useful in *E.faecium* because of the common occurrence of vancomycin resistance in these strains. Cephalosporins are inactive against enterococci except

ceftobiprole for *E.faecalis* infections . For the treatment of urinary tract infections single agent like nitrofurantoin, fosfomycin ,ampicillin or amoxicillin can be used.

Linezolide and Quinupristin/Dalfopristin are FDA approved drugs used in some VRE infections, both are bacteriostatic drugs and used as an alternative to the standard drugs .

VANCOMYCIN RESISTANT *ENTEROCOCCI* (VRE)

Epidemiology:

The first report of isolation of vancomycin resistant *E.faecalis* and *E.faecium* came from England in 1988 by Uttley et al[44] and thereafter from France also[11]. Now the emergence of vancomycin resistant *Enterococci* are being reported worldwide from many countries - European countries – Greece , Portugal , United kingdom and United States and also from Asia[7,11]. The presence of VRE was associated with the use of avoparcin- a glycopeptide used as a growth promoter in animal feeds and was banned from European countries. The rates of VRE is lower in Scandinavian countries and Netherlands due to strict adherence to infection control practices and in Latin America VRE rates ~ 4% [7]. Higher rates of VRE isolation in United States and Asia

could be attributed to higher usage of human antibiotics in these countries.

VRE now accounts for about 30% of all *Enterococcal* infections, the most common agent being *E.faecium* (>90%) (mand). Previous studies have reported that patients with VRE bacteremia were about 2.5 times more likely to die than those with VSE bacteremia indicating vancomycin resistance-a poor prognostic sign in critically ill patients[6]. The increased nosocomial VRE infection rates were mainly due to the increased VRE infections in ICUs(intensive care units) , however now the trend is also being observed in nonICU patients. VRE outbreaks in hospitals could be attributed to spread from patients colonized with VRE , however VRE have been isolated from hospital environment during outbreaks[11,45].

Previous studies[11,] conducted to assess community acquired VRE state that there is little evidence for significant transmission of VRE to healthy adults in the community. However the possibility of transmission to community members from patients colonized with VRE could not be denied when the colonized patients leave the hospital [11].

In India , vancomycin resistance among the *Enterococci* is emerging slowly. Previous studies have reported vancomycin

resistance in *Enterococci* ranging from 0-5.6% [2,22]. They also reported emergence of both Van A and Van B types, raising concern about the serious nature of the problem .

VANCOMYCIN RESISTANCE AMONG *ENTEROCOCCI:*

Vancomycin is one of the two glycopeptides currently in use to treat *Enterococcal* infections, the other one being teicoplanin. The mechanism of action of these drugs is inhibition of transglycolysation and transpeptidation of the pentapeptide units – the last step in peptidoglycan synthesis thereby interfering with the cell wall synthesis in bacteria[6].

VRE strains are classified based on phenotypic and genotypic characteristics as seven types of glycopeptides resistance – Van A , Van B, VanC, VanD , VanE, VanG and VanL [45]. Some of them have subtypes also. The clinically relevant phenotypes are VanA and VanB and are usually associated with *E.faecalis and E.faecium* and Van C is intrinsic to*E.gallinarum, E.casseliflavus.*. The genotype describes the gene clusters encoding the enzymes involved in the generation of structural components. These are the different peptidoglycan precursors that have decreased binding affinity for vancomycin ,

teicoplanin or both. These altered structural components result in phenotypic resistance.

The glycopeptide- susceptible strains have, in their cell wall , "D-alanyl-D-alanine" depsipeptide as the terminal end of peptidoglycan side chains. The antibiotic binds to this depsipeptide and there by inhibit the cell wall synthesis. In glycopeptide – resistant strains, this depsipeptide is replaced with " D-alanyl-D-lactate" or "D-alanyl-D-serine". These glycopeptide resistance types are described as follows,

VanA_ – it is encoded by Van A gene and is an inducible high level resistance to both vancomycin (MIC 64-1,000 µg/ml) and teicoplanin (MIC 16-512µg/ml). It is mediated the transposon *Tn1546* .The altered gene product is "D-alanine-D-lactate" . it is distributed in the following species *E.faecalis,E.faecium, E.avium, E.casseliflavus, E.durans, E.gallinarum, E.mundtii, E.raffinosus and E.sanguinicola.*

VanB – is an acquired inducible, variable level resistance to vancomycin (MIC 8-1,000µg/ml), but susceptible to teicoplanin (MIC 0.5-1µg/ml). The gene is located in plasmid and mediated by trasposons *Tn1547,Tn1549,Tn5382.* The gene product is "D-alanine-D-lactate". Ithas 3 subtypes (Van B1-B3) and is distributed in *E.faecalis,E.faecium, E.durans and E.gallinarum.*

Van C- encoded by constitutively expressed VanC gene located in chromosome and exhibits intrinsic low level resistance to vancomycin (MIC 2-32µg/ml) and susceptible to teicoplanin (MIC 0.5-1µg/ml). the end product is "D-alanine-D-serine" and has 4 subtypes distributed in *E.gallinarum* (C1) and *E.casseliflavus* (C2-C4).

Van D – encoded by Van D gene, constitutively expressed , chromosome mediated, moderate level resistance to vancomycin (MIC 64-128µg /ml) and susceptible / resistant to teicoplanin (MIC 4-64µg /ml) . The product is "D-alanine-D-lactate" and is found in *E.faecalis, E.faecium, E.avium* and *E.gallinarum.*

Van E, VanG and VanL – encoded by Van E,VanG , VanL genes located in chromosome, results in inducible intermediate level resistance to vancomycin (MIC 8-32µg/ml) , susceptible to teicoplanin (MIC 0.5-1µg/ml). The end product is "D-alanine-D-serine" and is found in *E.faecalis.*The identification of the different genotypes is crucial for therapeutic and infection control purposes.

RISK FACTORS associated with VRE [6,7,11,36,45]:

As the earlier reports of VRE were from ICUs, the risk factors associated with colonization and infection with VRE have been analyzed. The various risk factors are as follows:

- Presence of immunosuppression(haematologic malignancy,/bone marrow transplantation)
- Presence of Co-morbid conditions like diabetes, renal failure, high APACHE (Acute physiology and Chronic Health Evaluation) score, malignancy,
- Longer duration of hospital stay
- Intrahospital transfer to another ward
- Residence in a long term care facility
- Contact with another colonized /infected patient
- Invasive procedures
- Previous exposure to broadspectrum antibiotics – cephalosporins, vancomycin.
- Use of enteral tube feeding/ sucralfate
- Exposure to contaminated medical equipment
- The most important being exposure to heath care personnel nursing to a known VRE patient.

Colonization and Infection:

The first step in the infective process appears to be the colonization of the gastro intestinal tract. In most instances ,VRE isolation is from colonized patients than infected individuals. For every

one infected patient there could be as many as 10 colonizers[11].colonization usually involves gastrointestinal tract, perineal skin and rarely oral cavity and other sites.[11]

VRE infections usually occur in critically ill and debilitated hospitalized patients. The sites usually involved in VRE infections are bloodstream, intravascular catheters, surgical wounds, prosthetic devices, intra-abdominal sites and urinary tract. Various authors have reported mortality ranging from 46%-70% among patients infected with VRE[11]. The mortality is higher in patients with prolonged VRE bacteremia such as neutropenic patients , liver transplant recipients and seriously ill patients with co-morbid conditions like chronic renal failure. It is difficult to differentiate between colonization and infection, as mostly these infections are polymicrobial in nature and are recovered along with many known pathogens[45].

Source of infection and transmission of VRE:

The source of infection could be Endogenous – patients own Gastrointestinal tract in previously colonized individuals or Exogenous as contaminated environmental surfaces and ,medical devices –bed rails, linen,doorknobs,bed pans,stethoscope and blood pressure cuffs[6,11]. VRE are resistant to dessication and extreme temperatures and hence persists

for days to months . Contaminated food products may be a reservoir in non hospitalized individuals[11].

The most common mode of transmission is through the contaminated hands of healthcare workers in nosocomial VRE infections[11] and less commonly contact with contaminated equipments (clinical thermometers), and contaminated surfaces.

Laboratory Detection of VRE:

- Identification to species level, especially motility and pigment production differentiates the Van C phenotype (i.e) intrinsically resistant *E.gallinarum and E.casseliflavus* from others.
- Presumptive identification of VRE is done by vancomycin screen agars containing 6-8µg of vancomycin per ml of media.
- Confirmation is by determination of minimum inhibitory concentration of vancomycin and teicoplanin for the suspected VRE isolates.
- The genotypic expression can be detected by molecular methods – polymerase chain reaction for the amplification of vancomycin resistance genes- Van A, Van B and Van C, either conventional or multiplex PCR or by DNA hybridization probes.

Treatment of VRE infections:

The suggested therapeutic options for serious VRE infections are

- Combination therapy with high dose of cell wall active agents(ampicillin) and an aminoglycoside(if there is no acquired resistance observed for either of the agents)
- Linezolid – It is an FDA approved drug for treatment of VRE infections caused by both *E.faecalis, E.faecium* . It belongs to oxazolindones and it is a bacteriostatic drug. However it is recommended only as an alternative to other agents , but may play a crucial role in the treatment of meningitis and other CNS infections.
- Quinopristin-Dalfopristin – it is FDA approved, parentral semisynthetic streptogramin type A and B. it is also a bacteriostatic. It is active against *E.faecium* only and not active in *E.faecalis*
- Daptomycin- it is a lipopeptide active against both *E.faecalis* and *E.faecium* but it is not FDA approved. It is used as an alternative in situations of therapeutic failure of the commonly used agents.
- Other antimicrobials- Quinolones(ciprofloxacin and moxifloxacin), tetracyclines(doxycycline and minocycline), chloramphenicol can be used in combination therapy along with other agents.

PREVENTION AND CONTROL MEASURES[4]:

CDC's Hospital Infection control practices advisory committee has established certain guidelines and recommendations for prevention of VRE spread.

- Prudent use of vancomycin- inappropriate use of vancomycin is a risk factor for VRE colonization and infection and also emergence of vancomycin resistant *Staphylococci*. The medical staff should be educated about the appropriate or acceptable use of vancomycin(MRSA treatment, Severe antibiotic associated colitis as a second line agent , major surgical procedures involving implantation of prosthetic devices)
- Education of the personnel -special awareness should be created among all health care workers and patient care givers about the epidemiology and impact of VRE infections.
- Implementation of surveillance procedures (feces cultures) for early detection of VRE colonization .
- Infection control procedures aiming to limit cross contamination- isolation of known VRE patients and colonizers, strict adherence to hand washing.

MATERIALS AND METHODS:

Study Design : Cross sectional study

The present study was conducted in Department of Microbiology in Government Stanley Medical College and Hospital, Chennai, India.

Study Population:

Patients attending outpatient department and inpatients of Govt. Stanley Medical College and Hospital, Chennai.

Study Period:

July 2011 to November 2012- one year and 4 months

Materials :

A total of about21,045 clinical specimens such as urine, blood, pus, tissue fluids and feces obtained from all age groups of patients submitted to the microbiology laboratory , were analyzed and a total of 240 *Enterococcal* isolates recovered from these clinical samples were taken for further study.

The study was approved by our Institutional Ethical Committee .

METHODOLOGY:

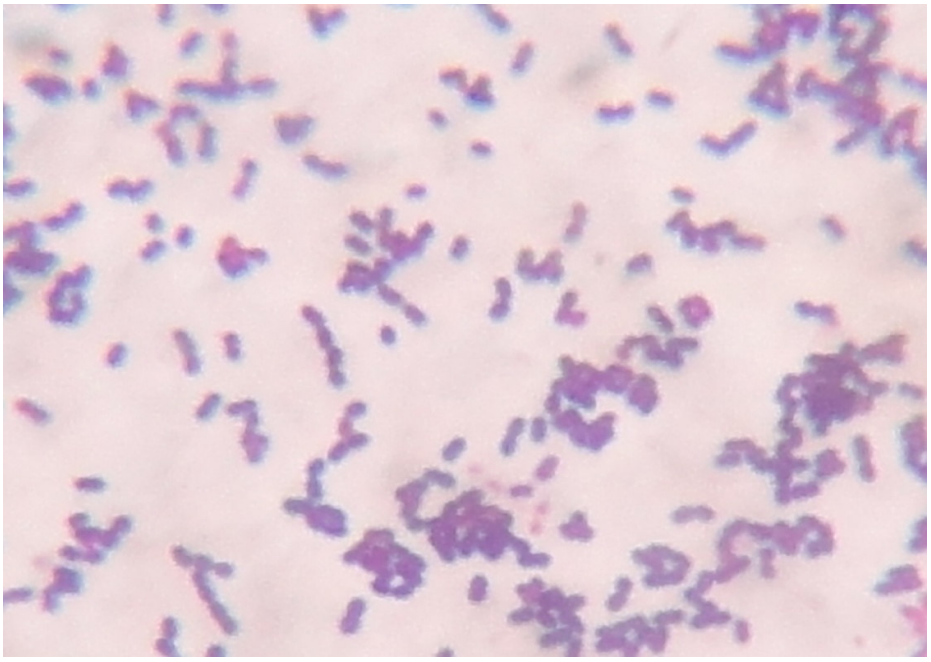
Collection and Processing of samples:

The various specimens such as urine, blood, wound exudates , pus and feces submitted to the microbiology laboratory from both inpatients and outpatients for bacteriological analysis were included in this study. The *Enterococcal* isolates grown from the above said samples were taken for further study and identified by standard techniques as follow.[6,7,8,28,45]

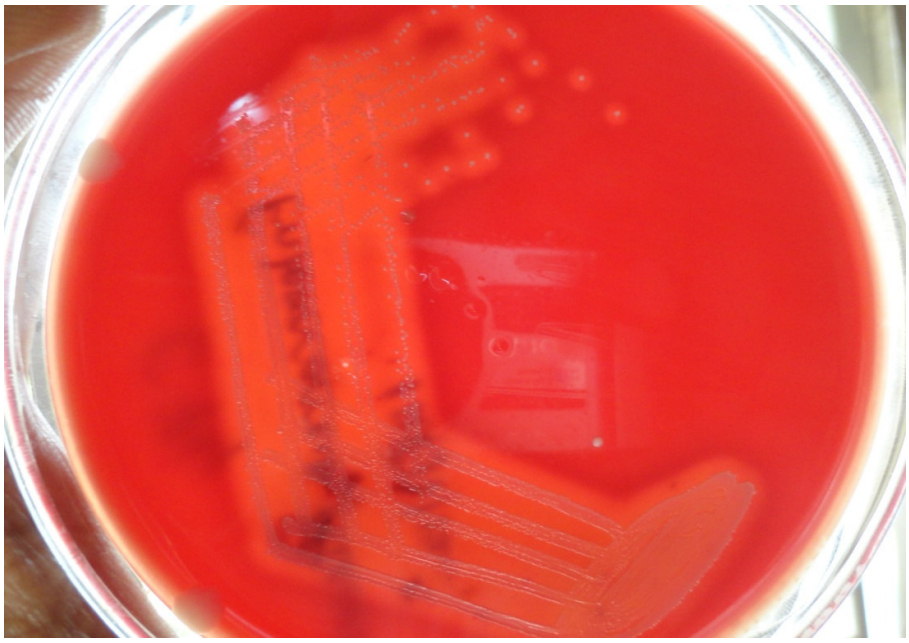
IDENTIFICATION OF THE GENUS:

The suspected *Enterococcal* isolates grown on the primary plating media such as blood agar and Mac Conkey agar were selected for further identification.

Preliminary tests for identification such as Gram stain, catalase test and motility test were performed on the selected isolates. Catalase negative, Gram Positive cocci in pairs and short chains were selected and processed further. For all the tests ATCC*Enterococcus faecalis 29212 is* included as a control strain.



GRAM STAINING- GRAM POSITIVE OVAL COCCI IN PAIRS & SHORT CHAINS-**ENTEROCOCCUS SPP.**



E.faecalis showing β hemolysis on human blood agar

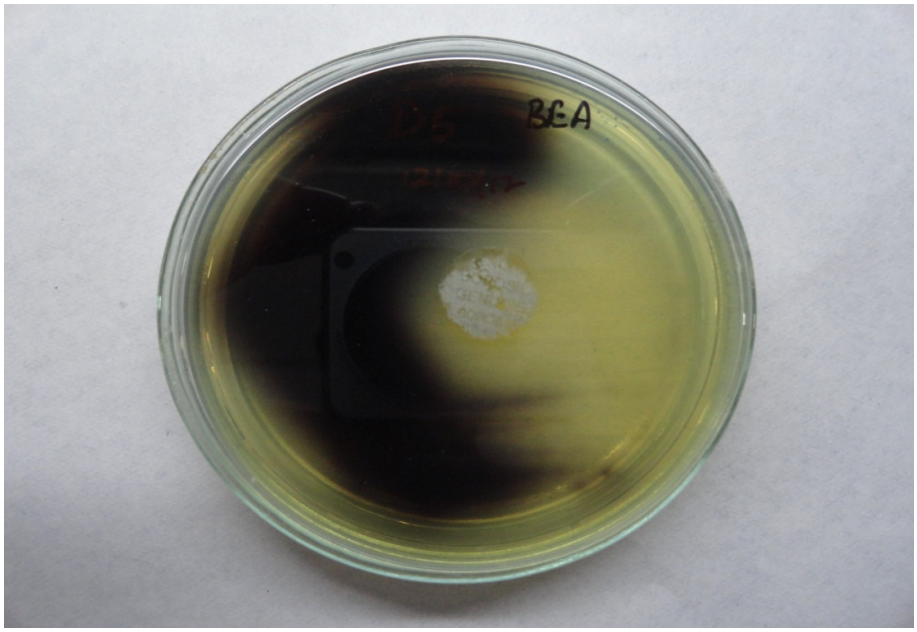
Bile esculin agar:

The suspected isolates were then inoculated onto Bile esculin agar (containing 40% bile) , incubated aerobically at 37ºC overnight. The next day the isolates showing black discoloration of the medium due to hydrolysis of esculin to esculetin were identified as BEA positive. After studying the colony morphology of each isolate plated on blood agar , MacConkey agar and bile esculin agar , the isolates which were nonhemolytic on blood agar, showing magenta pink colored tiny colonies on MacConkey agar and BEA positive were selected for further biochemical reactions.

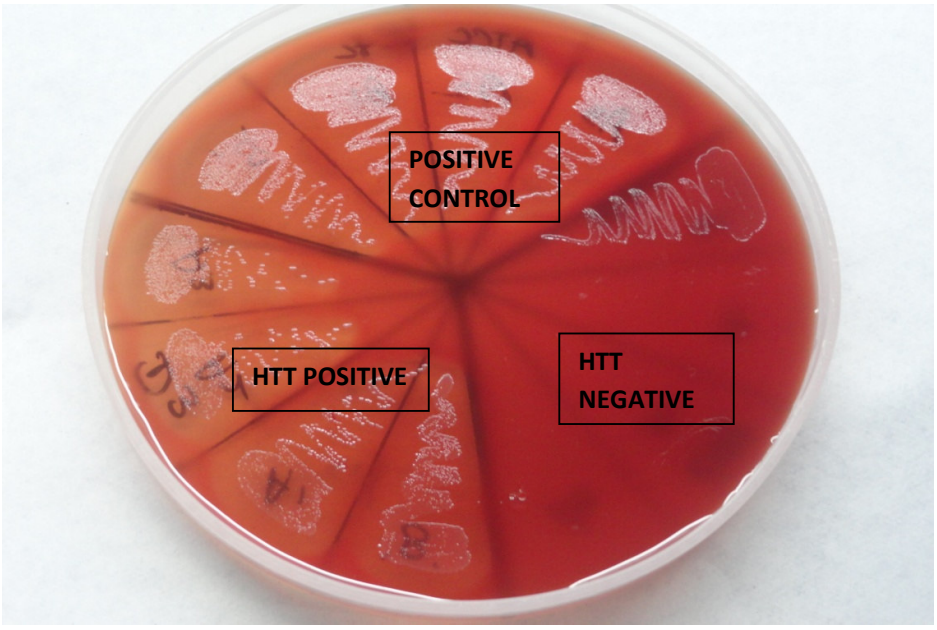
Heat tolerance test[11,38]:

The suspected *Enterococcal* isolates along with the control strains were tested for heat tolerance[11,38] by inoculating them into BHI broth and incubating them along at 60ºC for 30 minutes in a water bath. Subcultures from the broth were done on blood agar and MacConkey agar before incubation and at intervals of 10 min, 20min and 30 minutes after incubation . ATCC *E.faecalis 29212* was used as a positive control.

The growth of the positive control was checked before reading other isolates. The ATCC control strain has shows growth both before



BILE ESCULIN AGAR- blackish discoloration of medium-hydrolysis of esculin by Enterococcus spp.



HEAT TOLERANCE TEST

and after heating the broth at 60ºC for 30 minutes. The isolates showing growth before and after 30min of incubation at 60ºC were taken as heat tolerant *Enterococcal* isolates .

Salt tolerance:

Salt tolerant property of the suspected *Enterococcal* isolates were tested by inoculating 2 to 3 identical colonies of suspected isolates along with control strains into a tube containing nutrient broth with 6.5% sodium chloride and incubated at 37ºC for 24-72 hours. 1% bromocresol purple is added as an indicator to detect yellow discoloration on growth. The broth showing turbidity with or without yellow discoloration is taken as positive reaction and is confirmed by subculturing the broth on blood agar / MacConkey agar. The salt tolerant isolates grow well even in the presence of 6.5% NaCl.

Salt tolerant , BEA positive isolates, which were able to grow on MacConkey agar and at temperatures of > 45ºC were identified as *Enterococci* and selected for further speciation.

Speciation of the *Enterococcal* isolates was done based on the Faklam and Collins conventional identification scheme[20]. *Enterococci* were classified into the physiological groups I-V based primarily on

arginine dihydrolysis, fermentation of mannitol and sorbose . Further speciation was based on acid production from specific carbohydrates and motility and pigment production.

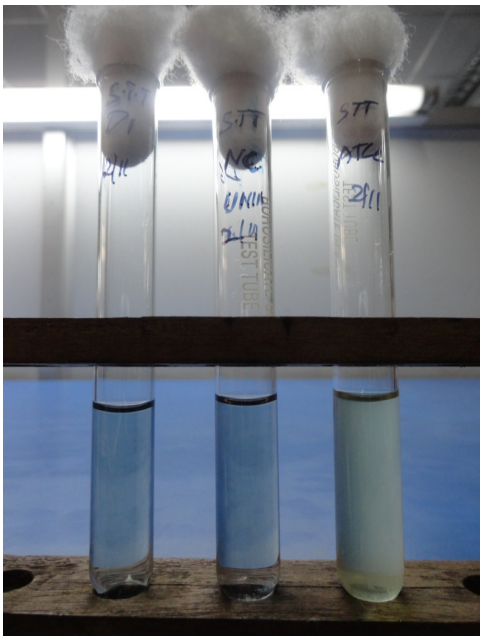
Arginine dihydrolysis:

Arginine dihydrolysis was tested by inoculating the isolate into a tube of Moeller's decarboxylase broth containing arginine and a control tube (without arginine), overlaid with sterile liquid paraffin and incubated for seven days at 37ºC . Control strains were also included in the test. Development of deep purple colour due to alkalinization after an initial change to yellow colour read as positive reaction. Persistant yellow color indicates negative reaction.

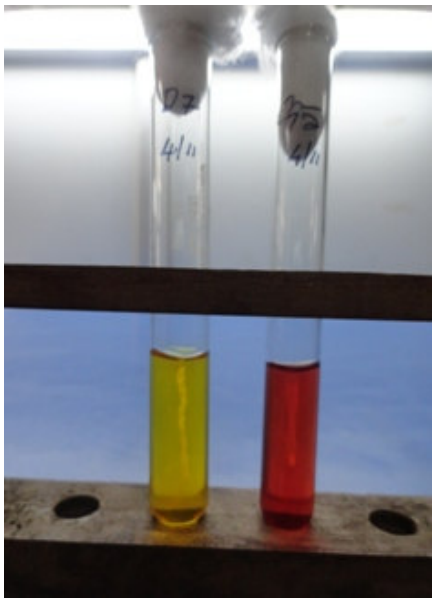
Mannitol motility medium:

The motility and fermentation of mannitol was tested by stab inoculating the isolates (including positive and negative control) into the medium and incubated at 37ºC overnight to detect the motility and acid production from mannitol.

To test the utilization of carbohydrates, the *Enterococcal i*solates were inoculated into carbohydrate fermentation medium containing 1% each of pyruvate, arabinose, sorbitol, sucrose, sorbose and raffinose and



SALT TOLERANCE TEST



MANNITOL MOTILITY MEDIUM

L-Mn+, non motile, R-Mn-,non motile

E.mundtii showing yellow pigment production

incubated at 37ºC overnight to detect acid production indicated by colour change to yellow.

Pigment production is tested by growing the isolate in blood agar and sweeping few colonies using a cotton tipped swab. Pigment production is indicated by yellow/orange tinge on the swab.[28]

The additional tests performed were production of black coloured colonies on 0.04% tellurite agar , which is a feature of *E.faecalis.*

***Enterococcus faecalis* – characteristics:**

Gram stain – gram positive cocci in pairs and short chains.

Catalase – Negative

Motility – Nonmotile

Colony morphology :

Blood agar (5% sheep blood agar) : α – or non hemolytic, small, cream colored,smooth colonies with entire edge. β- hemolysis is observed when human, horse or rabbit blood is used.

MacConkey agar : lactose fermenting, magenta coloured colonies.

Bile esculin agar – cases blackening of the medium- hydrolyses esculin in the presence of 40% bile.

Heat tolerance : survives a temperature of 60ºc for 30 minutes.

Salt tolerance : survive a salt concentration of 6.5% NaCl

Mannitol motility medium : nonmotile , ferments mannitol by producing acid.

Pigment production : pigment not produced.

Arginine dihydrolysis : hydrolyses arginine – produces deep purple colouration after initial colour change to yellow.

0.04% tellurite agar : produces black coloured colonies .

Carbohydrate utilization (1%) : ferments pyruvate and sorbitol, but not arabinose , sorbose.

Enterococcus faecium* - characteristics*:

Gram stain – gram positive cocci in pairs and short chains.

Catalase – Negative

Motility – Nonmotile

Colony morphology :

- Blood agar(5% sheep blood agar) : α – or non hemolytic, small, cream colored,smooth colonies with entire edge. β-hemolysis is observed when human, horse or rabbit blood is used.

- MacConkey agar : lactose fermenting, majenta coloured colonies.
- Bile esculin agar – cases blackening of the medium- hydrolyses esculin in the presence of 40% bile.

Heat tolerance : survives a temperature of 60ºc for 30 minutes.

Salt tolerance : survives salt concentration of 6.5% NaCl.

Mannitol motility medium : nonmotile , ferments mannitol by producing acid.

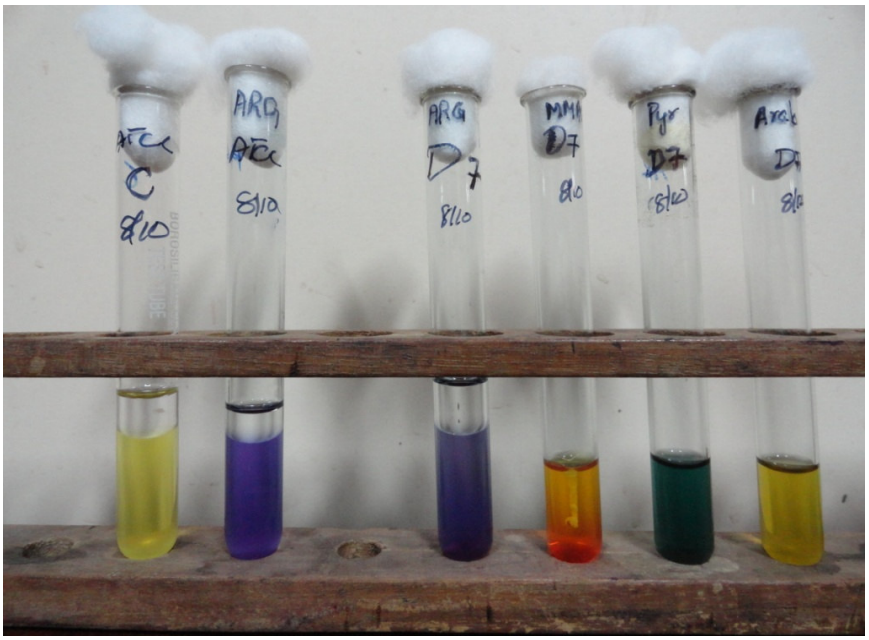
Pigment production : pigment not produced.

Arginine dihydrolysis : hydrolyses arginine .

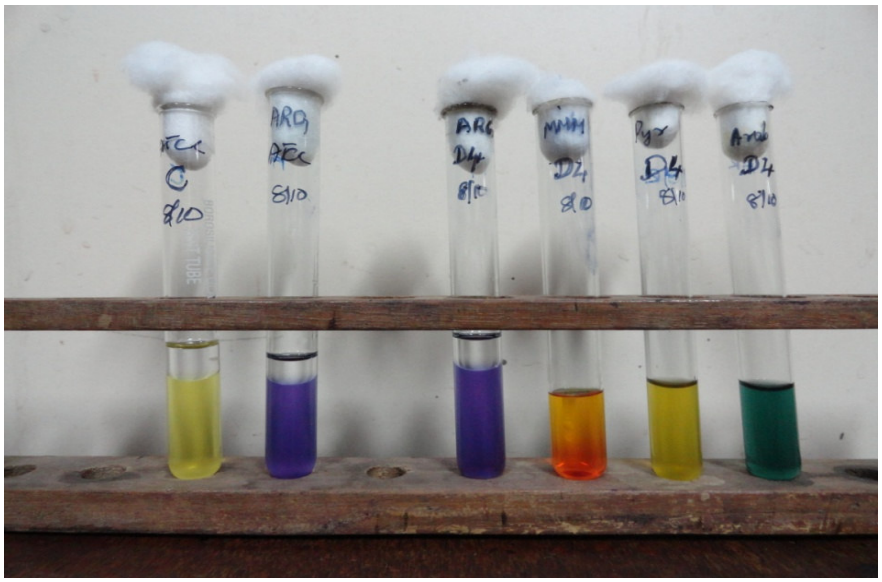
Carbohydrate utilization : ferments arabinose, sorbose –not fermented.

Others : Inherently resistant to Imipenem .

Other species of *Enterococci* were differentiated based on the following characteristics[8, 20,2845]



Biochemical reactions of E. faecium- ARG +, MMM +/-, PYR-, ARAB+



Biochemical reactions of E. faecalis- ARG +, MMM +/-, PYR+, ARAB-

Enterococcal species	**Arginine dihydrolysis**	**Mannitol Motility medium**	**Fermentation of sugars**	**Motility & Pigment production**
E.raffinosus Group I	Not hydrolysed	Fermented Nonmotile	Raffinose, Arabinose Sorbose fermented.	Nonmotile no pigment
E.sulfurous GroupIV	Not hydrolysed	Not fermented nonmotile	Raffinose ,arabinose fermented. Sorbose not fermented	Yellowish pigment produced.
E.columbae Group V	Not hydrolysed	Fermented nonmotile	Raffinose Arabinose fermented sorbose not fermented	No pigment
E.durans Group III	hydrolysed	Not fermented Nonmotile	Raffinose ,sucrose &, pyruvate not fermented.	
E.hirae GroupIII	hydrolysed	Not fermented nonmotile	Raffinose&sucrose fermented. Pyruvate not fermented	
E.dispar Group III	Hydrolysed	Not fermented Nonmotile	Raffinose sucrose &pyruvate fermented.	
E.avium Group I	Not hydrolysed	Fermented nonmotile	Arabinose,sorbose fermented, raffinose not fermented	
E.mundtii Group II	hydrolysed	Fermented nonmotile	Arabinose fermented	Yellow pigment produced

ANTIBIOTIC SUSCEPTIBILITY TESTING :

Antibiotic susceptibility testing of all the *Enterococcal* isolates and the screening and confirmatory tests for the detection of specific resistance mechanisms like Penicillin resistance , HLAR and Glycopeptide resistance were performed as per CLSI Standards[13,14].

ANTIBIOGRAM BY KIRBY-BAUER DISC DIFFUSION METHOD[13] :

The antibiotic susceptibility pattern of the isolates was determined by on Mueller –Hinton agar (MHA) .The bacterial inoculums is prepared by inoculating few identical colonies in a nutrient broth and incubated for 3-6hrs. It is standardized with 0.5 McFarland turbidity standard (1.5×10^8 CFU/ml) before inoculation , if the bacterial suspension is too thick it should be dilute to match the standard and if it is lees tubid ,it has to incubated further.

After standardization ,a sterile swabis dipped in that broth and the excess fluid is squeezed out by pressing on the side of the test tube, and it is streaked on the surface of the agar three times, turning the plate at 60º each time to produce a lawn culture of the organism.Then it is allowed to dry and the antibiotic discs are placed over the lawn culture within 15 minutes of inoculation.

The antibiotics tested were as follows – for urinary isolates penicillin 10 U, ciprofloxacin 5µg, doxycycline 30µg, high level gentamicin 120µg , high level streptomycin 300µg, vancomycin 30µg and teicoplanin 30µg . For isolates from other sites like pus, wound, blood –penicillin 10/ ampicillin 10µg,erythromycin 15µg, chloramphenicol 30µg, doxycycline 30µg, high level gentamicin 120µg , high level streptomycin 300µg, vancomycin 30µg and teicoplanin 30µg. All the materials and antibiotic disc were procured from Himedia laboratories Pvt.Ltd. Mumbai. The inoculated plates were incubated aerobically at 37ºC overnight. Next day the zone of inhibition of the bacterial growth around each disc is measured using ruler under reflected light except for vancomycin which should be read through transmitted light.The interpretation as susceptible, intermediate and resistant were done according to the CLSI guidelines[13].(Appendix)

SREENING TESTS FOR HIGH LEVEL AMINOGLYCOSIDE RESISTANCE (HLAR):

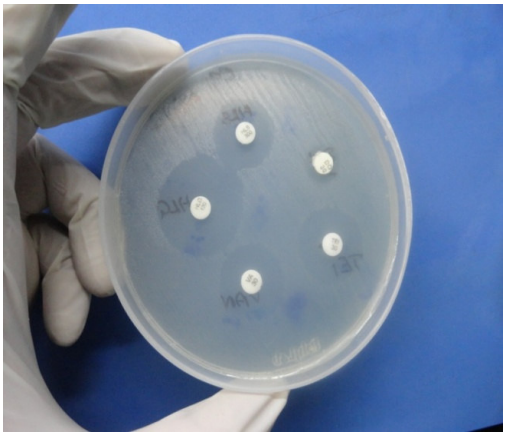
The *Enterococcal i*solates were screened for high level resistance to aminoglycosides using the antibiotic discs – high level gentamicin(HLG) 120µg and high level streptomycin(HLS) 300µg on Mueller – Hinton agar by standard disc diffusion method as described above

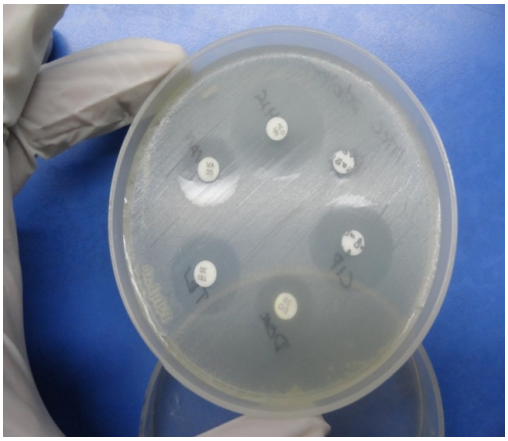
using McFarland 0.5 turbidity standard bacterial suspension and incubated at37ºC overnight.

The results interpreted as per CLSI standards[13] susceptible ≥10mm, inconclusive-7-9mm, resistant – 6mm.The test included *E.faecalis ATCC*29212 as negative control and *E.faeciumBM4147* positive control.The isolates showing zone of inhibition 7-9mm were tested again by agar dilution method using brain heart infusion agar (BHI) containing gentamicin 500µg /ml as recommended by CLSI guidelines.10 µl of the bacterial suspension after standardization with 0.5 Mc Farland Standard was spot inoculated onto the agar and incubated aerobically at 35±2ºC for 24-48hrs. in the same way Streptomycin 2000 µg/ml was also tested. The results were interpreted as resistant when > 1 colony is observed on the screen agar.

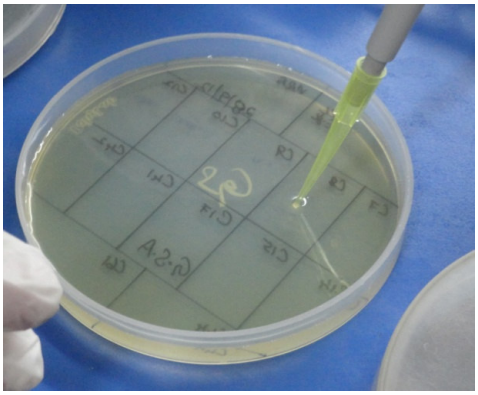
DETECTION OF VANCOMYCIN RESISTANCE :

Presumptive identification of vancomycin resistance can be done by Vancomycin screen agar (i.e) brain heart infusion (BHI) agar containing 6 µg /ml vancomycin. 10µl of 0.5 McFarland suspension of the isolate ,along with positive and negative control strains, is spot inoculated onto the agar surface and incubated aerobically **for 24hrs** at 35±2ºC.Growth of > 1 colony indicates presumptive vancomycin resistance which should be confirmed by determining the Minimum

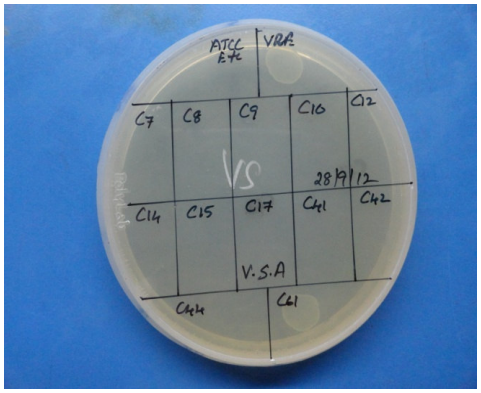




ANTIBIOTIC SUSCEPTIBILITY PATTERN- E.faecalis isolates



Spot inoculation of Enteroccoci on Vancomycin screen agar



E.faecium(C61) showing positive growth on vancomycin screen agar.

inhibitory concentration (MIC) for vancomycin[13]. The test included *E.faecalis ATCC*29212 as negative control and *E.faeciumBM4147* positive control

VANCOMYCIN MIC by BROTH MICRODILUTION METHOD[14]:

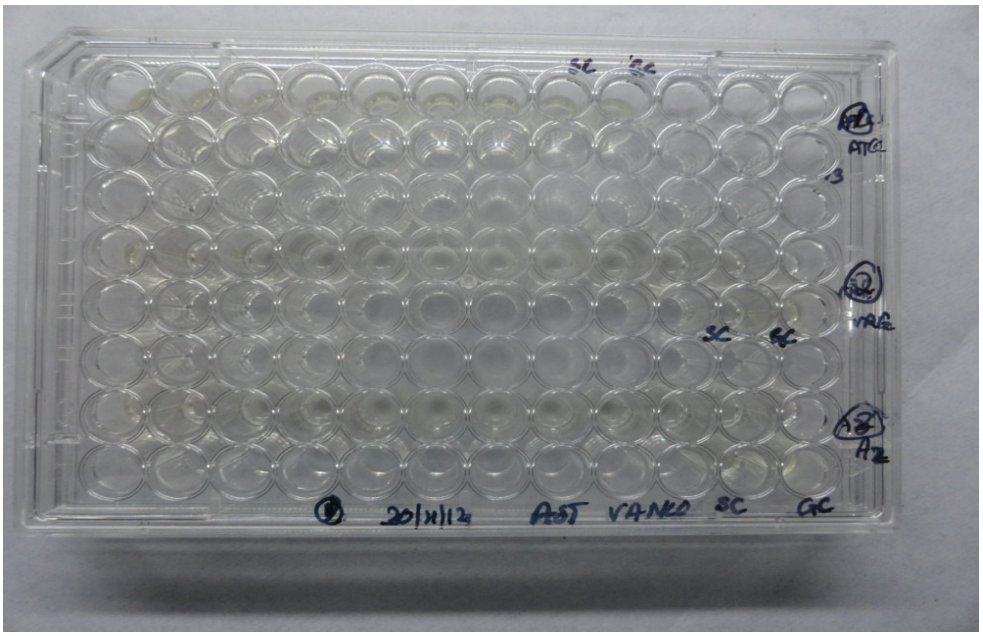
The minimum inhibitory concentration of vancomycin for the *Enterococcal* isolates grown on vancomycin screen agar were done as per the CLSI recommendations[14,44].

Preparation of drug concentrations of vancomycin;

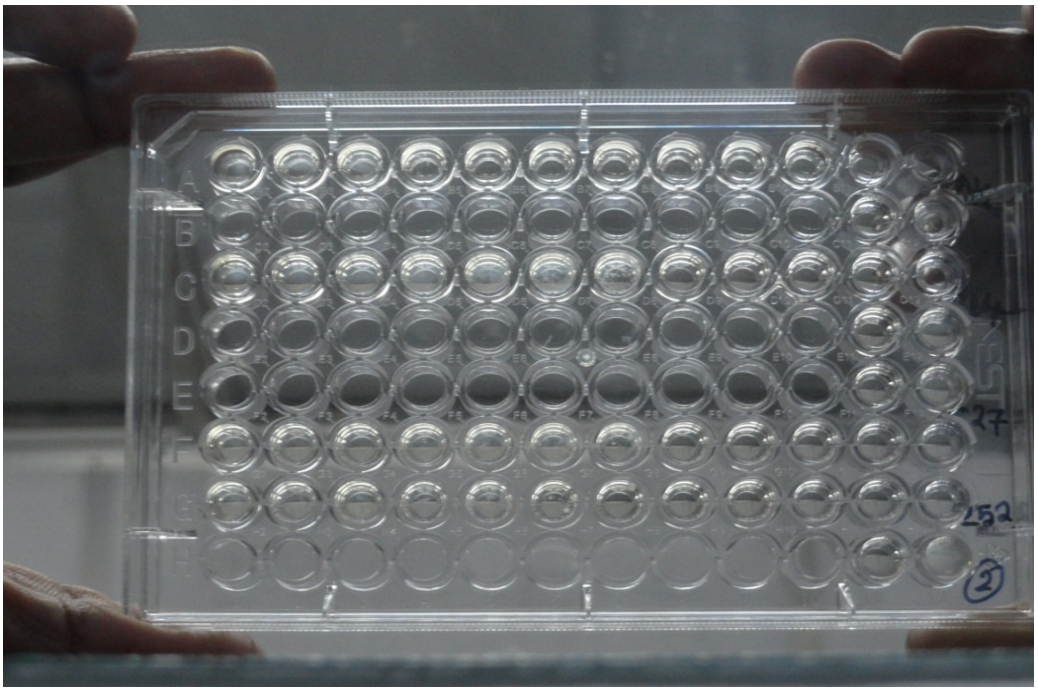
Stock solution is prepared as recommended in CLSI document on dilution testing[14].The vancomycin drug is dissolved in distilled water and the master dilution is prepared by diluting the required amount of drug in Cation adjusted Mueller-Hinton broth(CAMHB) (MHAbroth 2). Serial doubling dilution of the master dilution was perfomed in cation adjusted Mueller – Hinton broth .Thus the following concentrations of vancomycin 0.5µg /ml, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048µg/ml are prepared using CAMHB broth.

Bacterial Inoculum preparation :

Bacterial inoculum is prepared by **Direct Colony Suspension** method , and standardized with 0.5 McFarland standard. 1 in 100 dilution of this inoculum is prepared by adding 0.01 ml of this to 0.9 ml



Minimum Inhibitory Concentration(MIC) Testing of Vancomycin-Microbroth dilution technique.



Reading of Vancomycin MIC(Plane mirror reflection)

of CAMHB. 10 µl of this diluted suspension is equivalent to the final recommended inoculum of 5×10^4 CFU/ml , dispensed per well.

The serial doubling dilution of vancomycin already prepared is dispensed in 100µl amounts in the respective wells with a growth control(well containing CAMHB broth and bacterial inoculum, without vancomycin) and a sterility control (well containing CAMHB broth only). To this 10µl of bacterial inoculum was added to all wells except sterility control well and the plates were incubated at 35±2 ºC for **24 hrs** in ambient air. The test included *E.faecalis ATCC* 29212 as negative control and *E.faeciumBM4147* positive control QC strains.

Minimum inhibitory concentration (MIC) is the minimum concentration of the drug showing complete inhibition of bacterial growth visible to the naked eye. Results were interpreted after reading the MIC of QC strains which should fall within the recommended range. The MIC range of *E.faecalis ATCC* 29212 should fall within the range of 1-4µg/ml .The MIC of other isolates were interpreted as per CLS I guidelines[14].

MIC MINIMUM INHIBITORY CONCENTRATION FOR TEICOPLANIN:

The glycopeptide teicoplanin MIC was also tested in the same method as described above .The concentrations of Teicoplanin prepared were o.065µg/ml -0.125.0.5,1,2,4,8,16,32,64,128,256µg/ml by dissolving the teicoplanin in CAMHB broth in the same way as described in the CLSI guidelines[14].

The bacterial inoculum is prepared by direct colony suspension method and standardized with 0.5 McFarland turbidity standard. From this 1in 100dilution of the bacterial inoculum is prepared in the same way as described above. About 100µl of each drug concentration dispensed in to the wells of microtitre plate leaving the growth control well.10µl of the final inoculums is dispensed into all these wells except sterility control well. The plates were incubated at 37ºC for 24 hrs.

The MIC results were interpreted by noting the minimum concentration of the drug showing complete inhibition of the growth of the organism in the same way as described for Vancomycin.

MOLECULAR METHOD FOR THE DETECTION OF VRE:

Polymerase chain reaction (PCR) assay was performed by the for the detection of Vancomycin resistance genes in *Enterococci* especially in *E.faecium, and E.faecalis* by the PCR Kit procured from Helini Biomolecules,Chennai. The DNA is extracted from the *Enterococcal* isolates by using Helini Pure Fast Bacterial Genomic DNA Mini Spin Prep Kit and subjected to PCR and the gene product viewed by gel electrophoresis.

Extraction of DNA from the *Enterococcal* isolates :

1. 1.5 ml of overnight bacterial culture was taken and centrifuged at 12000 rpm at 4º C for 5 min.
2. To the pellet 180µl of Lysozyme digestion buffer was added
3. To this 20µl of Lysozyme was added and incubated for 10 min at 37 ºC.
4. Then 200µl of Lysis buffer was added.
5. Then20µl of proteinase K was added mixed well and incubated at 56ºC for 15 minutes in a waterbath
6. To this 200µl of Isopropanol was added and mixed well by inverting several times.

7. The entire volume of sample was transferred to Pure fast spin column using pipette and centrifuged for1min at 10,000rpm.
8. 500µl of wash buffer added to the spin column and centrifuged for 30 seconds and the flow-through was discarded , the spin column is placed back in collection tube.
9. Washing repeated twice using wash buffer II.
10. The flow- through is discarded mini spin centrifuged for 1 min to remove residual ethanol.
11. The spin column is placed in a fresh centrifuge tube and 50µl of pre warmed Elution buffer was added to the spin column , incubated for 2 min at room temperature , then centrifuged for 1 min.
12. Then the spin column is discarded DNA present in the centrifuge tube is stored at at -20º C.

5µl of this elute was used for PCR.

PCR master mix consists of dNTP mix 20Nm,Taq buffer and Taq polymerase enzyme

The Van A Primers designed by Gautham Pulavar, Helini Biomolecules, are as follows.

Forward Primer: 5'-TGCGCGGAATGGGAAAACGACA-3'

Reverse Primer: 5'-CAGCCCGAAACAGCCTGCTCAA-3'

The PCR Product size is 473bp representing Van A gene.

An optimal negative control was employed using 1 µl molecular grade water.

PCR amplification :

The PCR reactant mixture for each sample is prepared by adding 20µl of PCR Master Mix, 2µl of Van A Gene specific Primer mix , 5µl of Purified DNA of each sample and 3µl of Nuclease free water to a total final volume of 30µl.PCR amplification was performed in thermal cycler (MyGenie, Bioneer,South Korea) using the following thermal profile which consists of one cycle of initial denaturation at 95ºC for 4min followed by 35 cycle of denaturation at 95ºC for 30sec, primer annealing at 62ºC for 30sec, extension at 72ºC – 30sec and one cycle of Final extension at 72ºC for 5min.

Analysis of PCR product was done by agarose gel electrophoresis.

About 1.5% of agarose gel was prepared by mixing 0.75 grams of agarose powder in 50 ml of electrophoresis buffer and heated in a microwave oven till agarose is uniformly dissolved. After cooling to

56ºC, 5µl of Ethidium bromide was added using gloved hands. Ethidium bromide is carcinogenic and hence should be handled with gloved hands and the tip is discarded into the black bin. After cooling the solution, it is poured into a gel casting tray containing comb and allowed to solidify. After hardening , the gel is placed in the electrophoresis tank. The electrophoresis buffer provided in the kit is diluted ten times and is poured into the tank till the gel is completely immersed, then the comb was carefully removed. Then the electrical leads were connected to the electrophoresis tank. About 15µl of loading buffer containing the PCR product and the tracking dye is loaded into each well using micropipette. About 10µl of 100 bp DNA ladder was loaded into the first well followed by 15µl of the sample in the other wells. A constant current of 100 volts was applied and the gel is allowed to run till the tracking dye reaches three fourth of the gel.

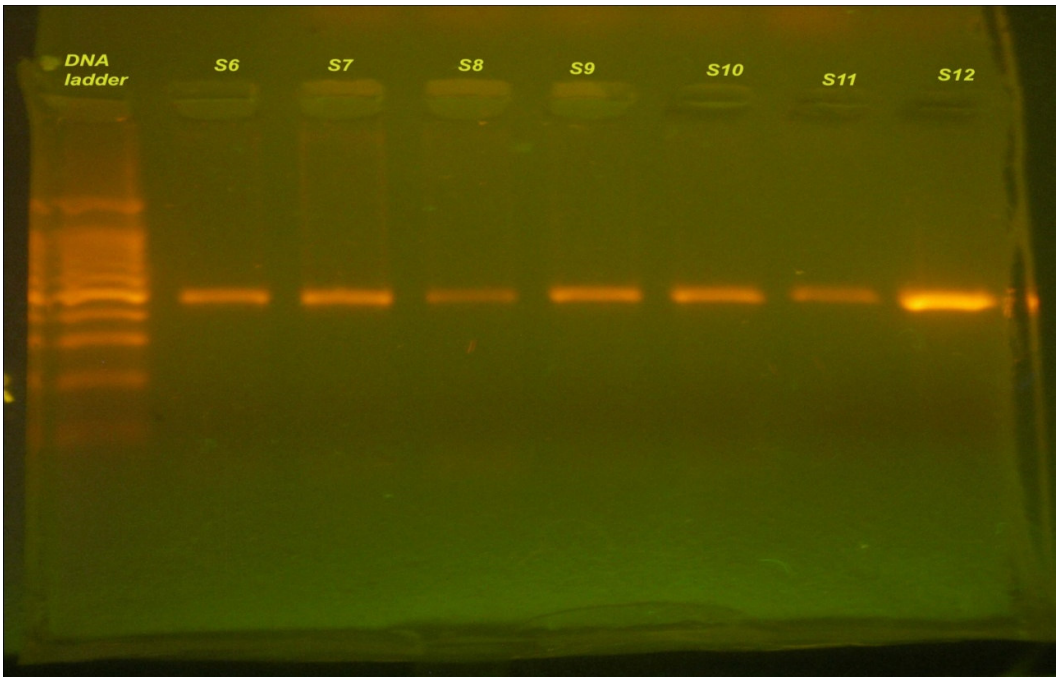
Then the gel tray is removed from the tank . Then the gel is removed from the tray and placed in the UV transilluminator for observation of bands of 473 bp size .

POLYMERASE CHAIN REACTION-Detection of vanA genotype

DNA extraction-Minispin column

Thermal cycler-PCR amplification

UV transilluminator



PCR product – Agarose gel image, S12-positive control-van A, E.faecium, S6-11- test isolates showing band of 473 bp size

OBSERVATIONS AND RESULTS:

This study was carried out in the department of Microbiology during the 18 month period from July 2011 to November 2012 . A total of about 8774 urine specimens,6305 pus specimens, 3655 blood specimens,2183 tissue fluid specimens and 128 feces specimens were analyzed for *Enterococcal* growth. The results were analyzed as follows.

A total of about 240 *Enterococcal* isolates were recovered from the above samples, of which majority were from urine specimens-211, 13 from blood specimens, 8 from pus specimens , 2 from tissue fluids (bile, Bronchial secretion) and 6 isolates from feces specimens.

Table .1.Distribution of samples showing growth of *Enterococci*

Specimen type	OP		IP		Total	
	No of samples analyzed	Positive for *Enterococcal* growth	No of samples analyzed	Positive for *Enterococcal* growth	No of samples analyzed	Positive for *Enterococcal* growth
Urine	4169	113 (2.7%)	4605	98 (2.1%)	8774	211 (2.4%)
Pus	586	-	5719	8(0.001%)	6305	8 (0.001%)
Blood	319	-	3336	13 (0.003%)	3655	13 (0.003%)
Tissue fluids	3	-	2180	2 (0.009%)	2183	2 (0.009%)
Feces	60	-	68	6 (8.8%)	128	6 (4.6%)
Total	5137 (24%)	113 (2.1%)	15908 (76%)	127 (0.007%)	21045	240 (1.1%)

OP- outpatients, IP – inpatients

The majority of the specimens were from inpatients (76%) than from outpatients(24%) . Majority of the *Enterococcal* isolates were from urine specimens 211 (87%) , followed by blood (5%),pus (3%) tissue fluids (0.8%),feces(4.6%).

Table 2: Distribution of *Enterococcal* isolates

Specimens	ICU/ IMCU	Non ICU					Total
		nephrology	urology	surgery	medicine	pediatrics	
Urine	9	11	92	12	38	47	211
Blood	4	-	-	-	7	2	13
Pus	-	-	-	8	-	-	8
Tissue fluids	1	-	-	-	1	-	2
Feces	-	-	-	-	4	2	6
Total	14(6%)	11(4.5%)	92(38%)	20(8%)	50(21%)	51(21%)	240
	ICU-14(6%)	Non ICU –226(94%)					

ICU- Intensive care units, IMCU-Intensive medical care unit.A total of 14 (6%) *Enterococci* isolates were from intensive care units (medicine, surgery). The isolates from various specialties were 11(4.5%) from nephrology, 92(38%) from urology, 20(8%) from surgery , 50(21%) from medicine , 51(21%) from pediatrics.

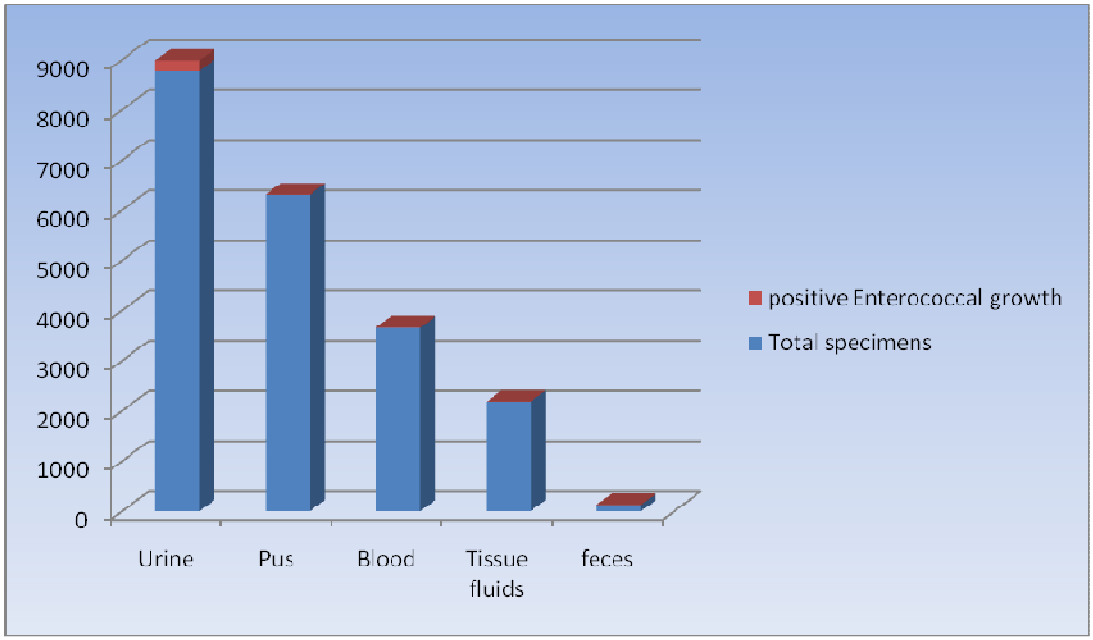


Figure 1 .DISTRIBUTION OF SPECIMENS POSITIVE FOR GROWTH OF ENTEROCOCCUS SPP.

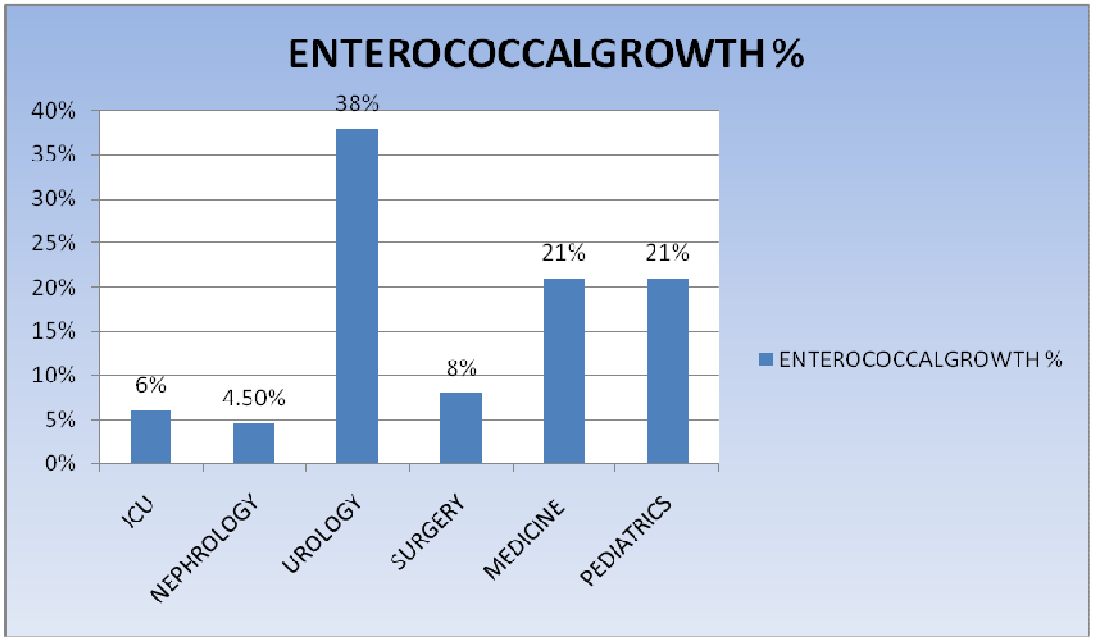


Figure 2 DISTRIBUTION OF ENTEROCOCCAL GROWTH AMONG DIFFERENT SPECIALITIES

Table 3.Age and sex distribution of *Enterococcal* isolates .(n-240)

Age	sex		Total
	Male	Female	
Adults(≥13yrs)	75 (40%)	114 (60%)	189 (79%)
Children(≤12yrs)	27 (53%)	24 (47%)	51 (21%)
Total	102 (43%)	138 (57%)	240

Out of the total 240 *Enterococci* isolated, majority were isolated from adult patients189 (79%),however around 51(21%)of isolates from pediatric patients.

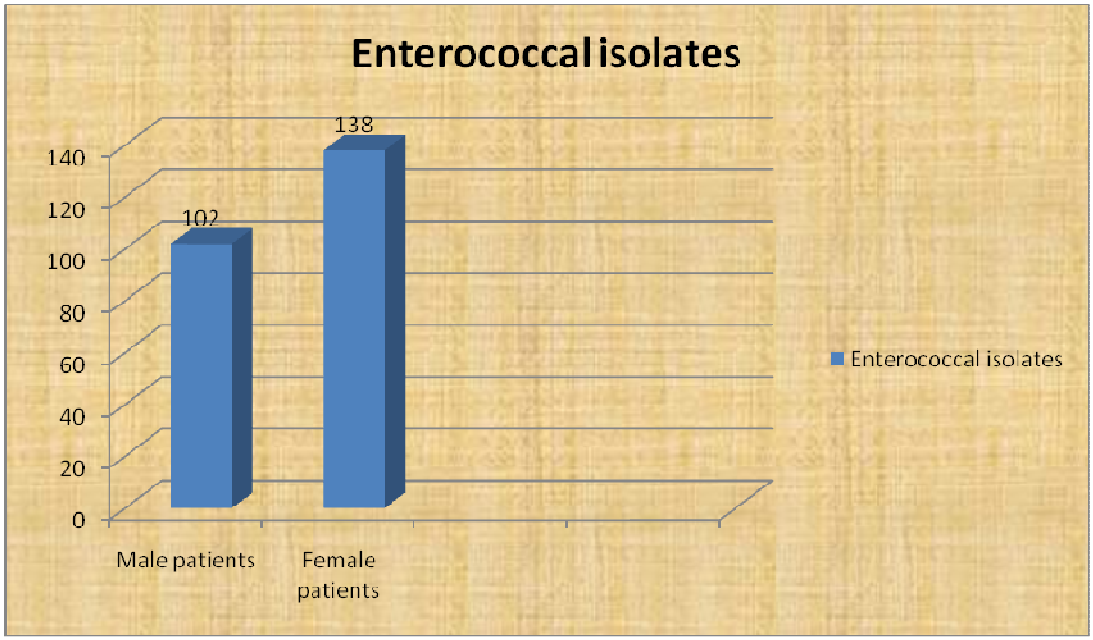


Figure 3 Distribution of *Enterococcal i*solates among male and female patients

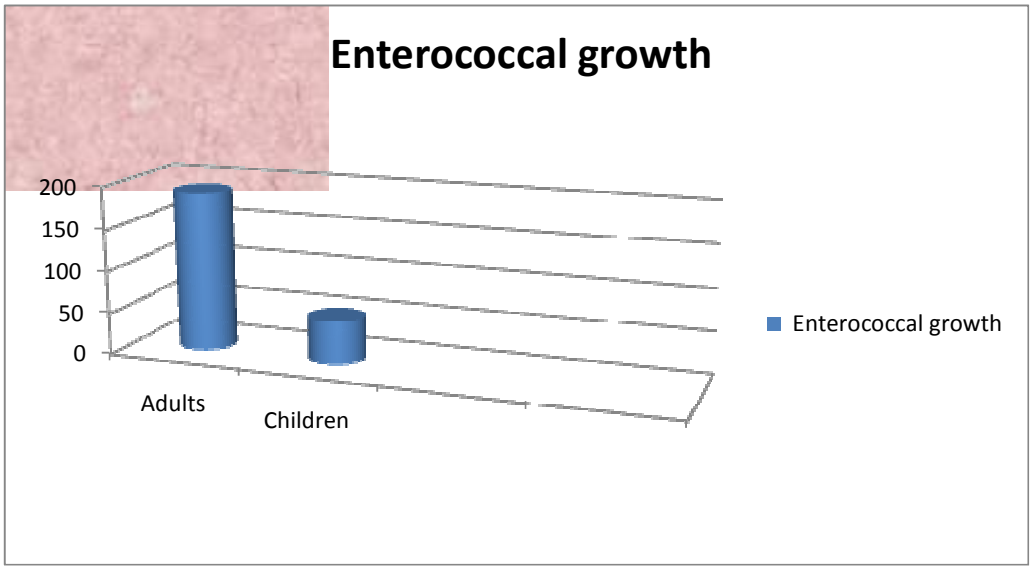


Figure 4 Distribution of Enterococcal Growth among Adults & childrens

Table 4. Distribution of different *Enterococcal* species among the specimens.

Enterococcal Species	Urine	Blood	Pus	Tissue fluids	Feces	Total no	Prevalence value per 100subjects
E.faecalis	115	6	2	-	3	126 (52.5%)	52.5%
E.faecium	72	5	2	2	3	84 (35%)	35%
E.raffinosus	8	-	-	-	-	8(3%)	3%
E.sulfurous	5	-	-	-	-	5(2%)	2%
E.columbae	3	1	-	-	-	4(1.6%)	1.6%
E.CDC PNSE2	3	-	-	-	-	3(1.2%)	1.2%
E.durans	1	-	1	-	-	2(0.8%)	0.8%
E.hirae	2	-	-	-	-	2 (0.8%)	0.8%
E.dispar	1	1	-	-	-	2(0.8%)	0.8%
E. asini	1	-	1	-	-	2(0.8%)	0.8%
E.avium	-	-	1	-	-	1(0.4%)	0.4%
E.mundtii	-	-	1	-	-	1(0.4%)	0.4%
Total	211	13	8	2	6	240	

E.faecalis is the predominant species followed by *E.faecium*. Other *Enterococcal* species such as *E.sulfurous, E.columbae* , have been isolated from urine samples and *E.columbae,E.dispar* from blood,*E.mundtii,E,asini,E.avium and E.durans* from pus samples .

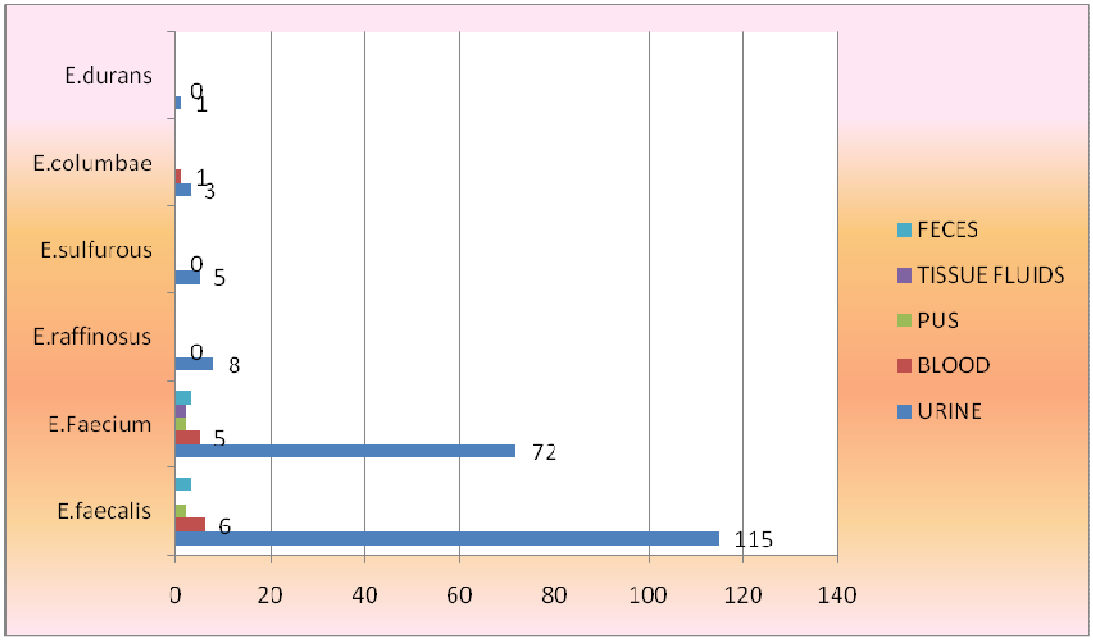


Figure 5. Distribution of different Enterococcal species among the specimens

Table 5. Antibiotic susceptibility pattern of Predominant *Enterococcal* species by Kirby-Bauer disc diffusion method.

Enterococcal species	Total no	Penicillin G		Ampicillin		Doxycycline		ciprofloxacin		High level Streptomycin		High level gentamicin		Vancomycin	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R
E.faecalis	126	11 9%	115 91%	110 87%	16 13%	45 36%	81 64%	14 11%	112 89%	82 65%	46 35%	68 54%	58 44%	122 97%	4 3%
E.faecium	84	11 1%	83 99%	18 21%	66 79%	27 32%	57 68%	7 8%	67 92%	51 61%	33 39%	26 31%	58 69%	62 74%	22 26%
E.raffinosus	8	0 0%	8 100 %	3 38%	5 62%	7 88%	1 22%	5 62%	3 38%	4 50%	4 50%	7 88%	1 22%	7 88%	1 22%
E. sulfurous	5	2 40%	3 60%	3 60%	2 40%	5 100%	0 0%	2 40%	3 60%	5 100 %	0 0%	5 100%	0 0%	5 100%	0 0%
E.durans	3	2 67%	1 33%	3 100%	0 0%	3 100%	0 0%	2 67%	1 33%	2 67%	1 33%	3 100%	0 0%	3 100%	0 0%
E.CDC PNSE2	3	2 67%	1 33%	3 100%	0 0%	2 67%	1 33%	1 33%	2 67%	2 67%	1 33%	2 67%	1 33%	2 67%	1 33%

The results were interpreted by measuring the zone of inhibition of growth around each disc as per CLSI guidelines[13]. Most of *E. faecalis* isolates are resistant to penicillins (91%) but preserve sensitivity to ampicillin(87% - sensitive), susceptibility to doxycycline is around 36% and ciprofloxacin 11%.The *E .faecium* isolates are resistant to penicillin(99%), ampicillin (79%) doxycycline(68%) and ciprofloxacin (92%).

Table 6.HLAR (High Level Aminoglycoside Resistance) among *E.faecium and E.faecalis* isolates by Disc diffusion method

Enterococcal species	Total isolates	Resistant to both HLG (120µg) & HLS(300µg)	Resistant to HLS only	Resistant to HLG only	Total HLAR
E.faecalis	126	18	37	31	86(68%)
E.faecium	84	24	33	11	68(80%)
Total	210	42(20%)	70(33%)	42(20%)	154(73%)

HLG-high level Gentamicin(120µg),HLS-high level Streptomycin (300µg)

Pvalue for E.*faecalis* HLAR -86/126 = 68 per 100 subjects, Pvalue for *E,faecium* HLAR -68/84 = 80 per 100 subjects

The results were interpreted by measuring the zone of inhibition around the antibiotic disc as per CLSI guidelines[13] . The total HLAR-high level aminoglycoside resistance observed is 73% and resistance to both agents observed in a total of 42 isolates (20%) and resistance to streptomycin only is observed in a total of70 isolates (33%) and resistance to gentamicin only is observed in42 isolates (20%).The

HLAR is observed in *E.faecalis* isolates was 68% and about 80% in *E.faecium.*

Then all the 240 *Enterococcal* isolates(irrespective of the susceptibility pattern of vancomycin by disc diffusion method), were screened on vancomycin screen agar(containing vancomycin 6µg/ml) for presumptive identification of vancomycin resistance. About 29 isolates showed growth indicating resistance to vancomycin.These 29 isolates were subjected to MIC (Minimum Inhibitory Concentration) for vancomycin as a confirmatory test. Of these 29 isolates, 8 isolates turned out to be susceptible by vancomycin MIC (≤4µg/ml) and 11 isolates showed vancomycin MIC values 8-16µg /ml ,and 10 isolates ≥32µg/ml hence interpreted as Vancomycin Resistant *Enterococcal* isolates as per CLSI guidelines[14] .(Appendix)

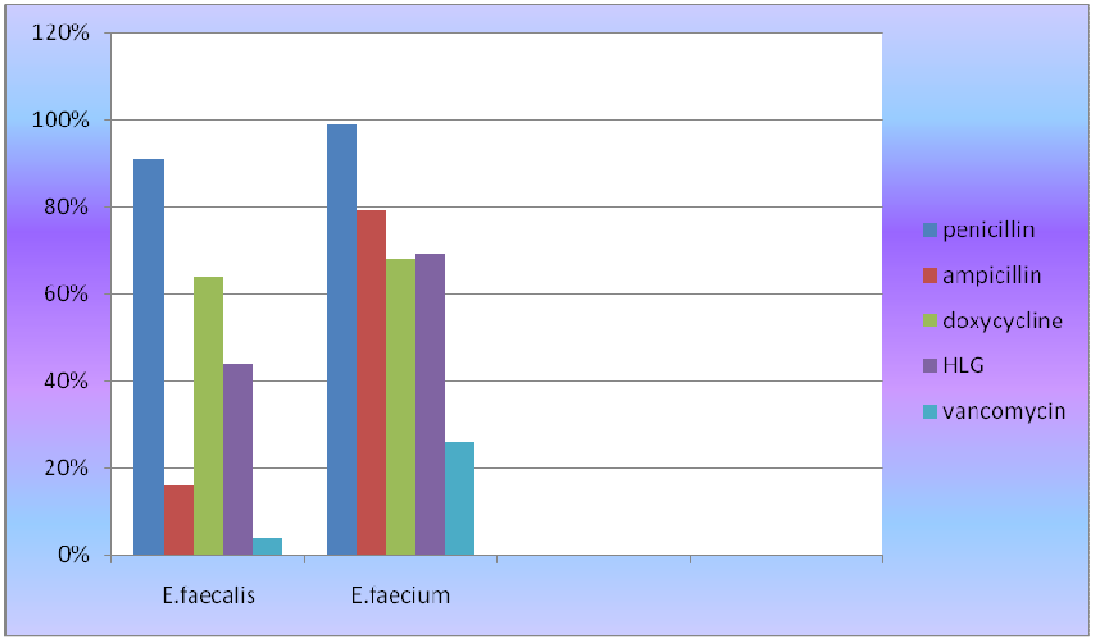


Figure 6Antimicrobial resistance pattern of *E.faecalis* and *E.faecium*

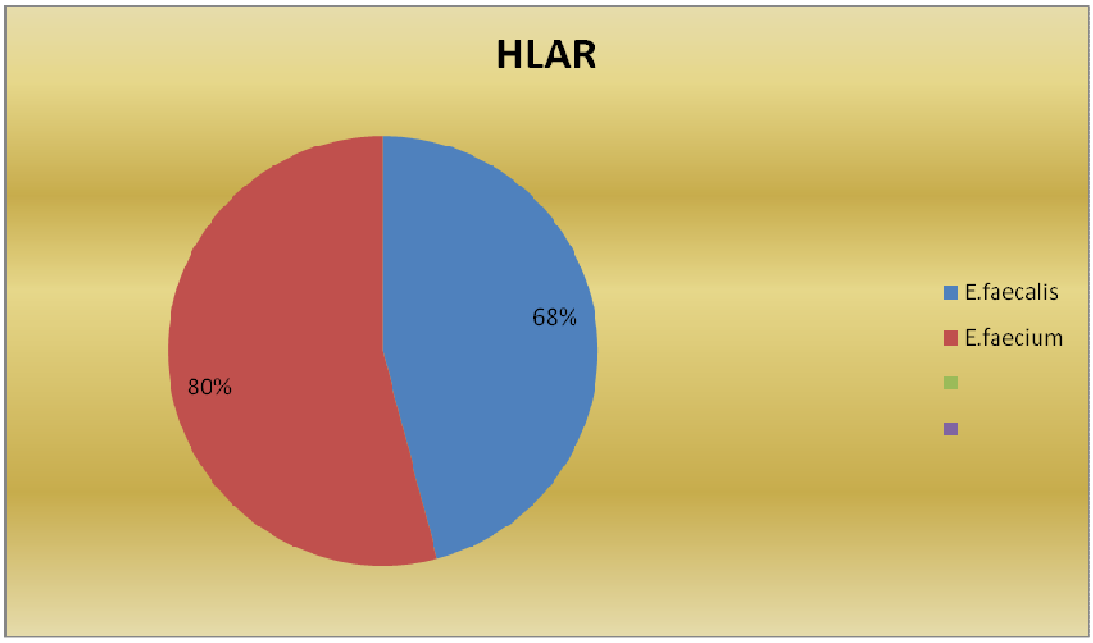


Figure 7. High level Aminoglycoside Resistance-Enterococci

Table.7.MIC values of Vancomycin for the VRE isolates.

VRE isolates n-21	Vancomycin MIC values (µg/ml)							Total
	Intermediate (8-16 µg/ml)		Resistant (≥32 µg/ml)					
	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	256 µg/ml	512 µg/ml	
E.faecium	2	6	1	4	2	2	1	18
E.faecalis	-	1	1	1	-	-	-	3
Total	2	7	2	4	2	2	1	21

MIC- Minimum inhibitory concentration

Out of the total 21 VRE isolates, the MIC values of 11 isolates fall within the intermediate range 8-16µg/ml, and10 isolates fall within the resistant range of 32-512µg/ml, interpreted as per CLSI guidelines[14] (Appendix)

Table 8.MIC values of teicoplanin for the VRE isolates

VRE isolates n-21	Teicoplanin MIC values (µg/ml)								Total
	Susceptible(≤8 µg/ml)					Intermediate (16 µg/ml)	Resistant (≥32 µg/ml)		
	0.5 µg/ml	1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	
E.faecium	7	2	-	-	-	4	4	1	18
E.faecalis	0	2	-	-	-	1	-	-	3
Total	7	4	-	-	-	5	4	1	21

MIC- Minimum inhibitory concentration

Out of the total 21 VRE isolates, the teicoplanin MIC of 11 isolates fall within the susceptible range (0.5-1 µg/ml) and 5 in intermediate range-16 µg/ml and 5 isolates fall within the resistant range(>32 µg/ml interpreted as per CLSI guidelines[14] (Appendix).

Table 9.Phenotypic classification of VRE isolates based on MIC Interpretation of Vancomycin and Teicoplanin .

Van phenotype	*E.faecium*	*E.faecalis*	Total
Van A vancomycin MIC ≥64 µg/ml(R) & teicoplanin MIC ≥16µg/ml(R)	9(50%)	1 (8.3%)	10(48%)
Van B Vancomycin MIC ≥8 µg/ml (IM/R) & teicoplanin MIC 0.5-1 µg/ml (S)	9 (50%)	2(15.3%)	11 (52%)
Total VRE isolates	18(72%)	3 (12%)	21

Prevalence rate of total VRE (21/240) - 8.75 per 100 subjects.

As per the above results, the vanA phenotype showing resistance to both vancomycin(≥64 µg/ml) and teicoplanin(≥16 µg/ml) was observed in 10 VRE isolates (10/21) (48%). About 11 isolates belong to VanB (11/21) (52%) (phenotype with vancomycin MIC ≥8 µg/ml including the intermediate and resistant range and teicoplanin MIC in the susceptible range usually (0.5-1 µg/ml) interpreted as per CLSI guidelines[14,45] (in appendix)

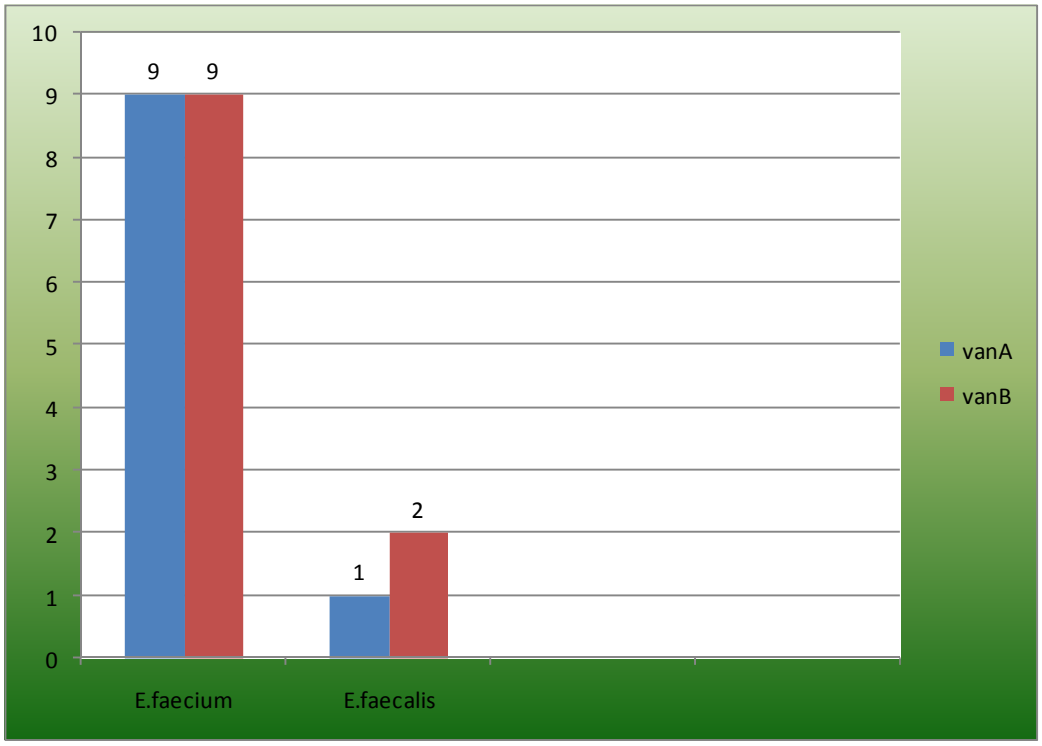


Figure 8 Phenotypic classification of Vancomycin Resistance types in Enterococci

All the 21 vancomycin resistant Enterococcal isolates were subjected to PCR assay to detect the presence of Van A gene and the results were as follows.

Table 10. Distribution of Van A Genotype in the VRE isolates

Enterococcal isolates n-21	Total isolates tested	VanA genotype	
		present	Absent
E.faecium	18	9 (50%)	9(50%)
E.faecalis	3	1 (33.3%)	2(66.6%)
Total	21	10(48%)	11(52%)

Out of the total 21 VRE isolates 1 *E.faecalis* and 9 *E.faecium* isolates were of vanA genotype showing bands of 473bp as per the PCR assay for vanA gene . The remaining 11 VRE isolates did not show any band corresponding to vanA gene They should be evaluated further to assess their genotype.

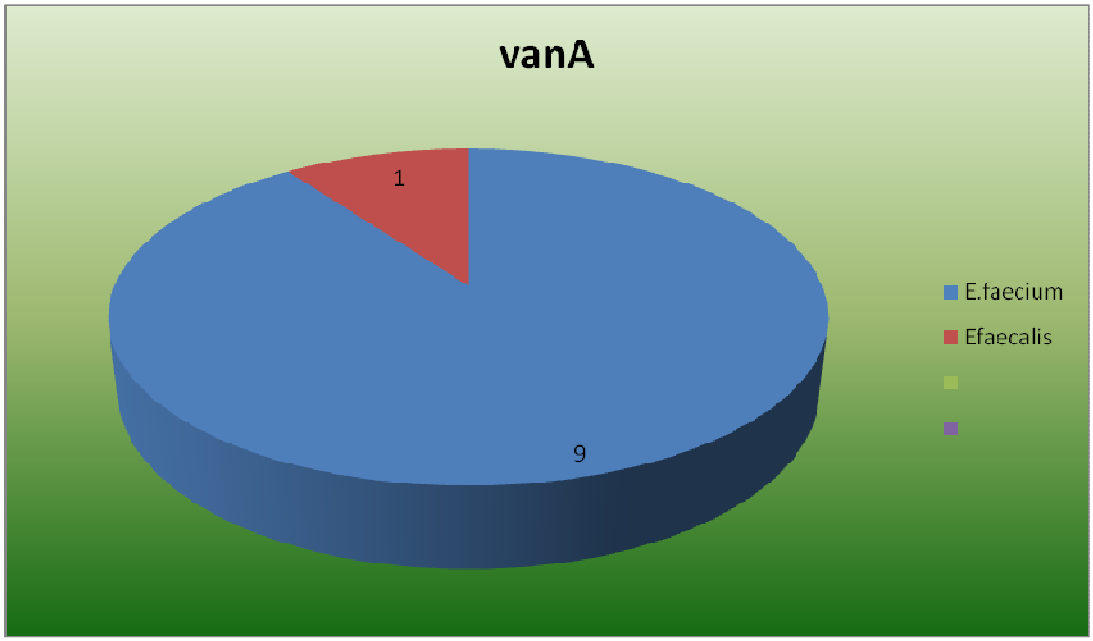


Figure 9.Distribution of VanA Genotype among the VRE isolates

Table 11. The correlation of MIC values of vancomycin and teicoplanin with Genotyping for Van A gene .

VRE isolates	No of isolates	MIC for Vancomycin µg/ml	MIC for Teicoplanin µg/ml	Presence of van A gene
E.faecium	9	64- 512 µg/ml	16-64µg/ml	9
E.faecalis	1	64 µg/ml	16 µg/ml	1
Total	10	≥64µg/ml	≥16 µg/ml	10

About 9 *E.faecium* isolates and 1 *E,faecalis* shows High level resistance to both Vancomycin and Teicoplanin and are of VanA phenotype

Thus there is 100% concordance of phenotypic classification by Vancomycin MIC and genotypic detection of the VanA resistance type.

Table . 12- Antibiotic susceptibility pattern of VRE isolates to supplemental drugs by Kirby- Bauer disc diffusion method.

Enterococcal species	Total no	LZ		CK		RP	
		S	R	S	R	S	R
E.Faecalis	3	3 100%	0 0%	0 0%	3 100%	NA	NA
E.faecium	18	18 100%	0 0%	7 39%	11 61%	18 100%	0 0%
Total	21	21 100%	0 0%	7 33%	14 67%	18 100%	0 0%

LZ-Linezolid, CK-chloramphenicol, RP-pristinamycin (Quinupristin / Dalfopristin), NA- not applicable(*E.faecalis* is inherently resistant to Q/D).

All of the VRE isolates were susceptible to Linezolid(100% sensitivity), whereas the 3 *E.faecalis* were resistant to chloramphenicol . Out of the 18 *E.faecium* VRE isolates, 11(61%) showed resistance to chloramphenicol and all of the *E. faecium* VRE isolates were sensitive to Quinupristin/Dalfopristin.

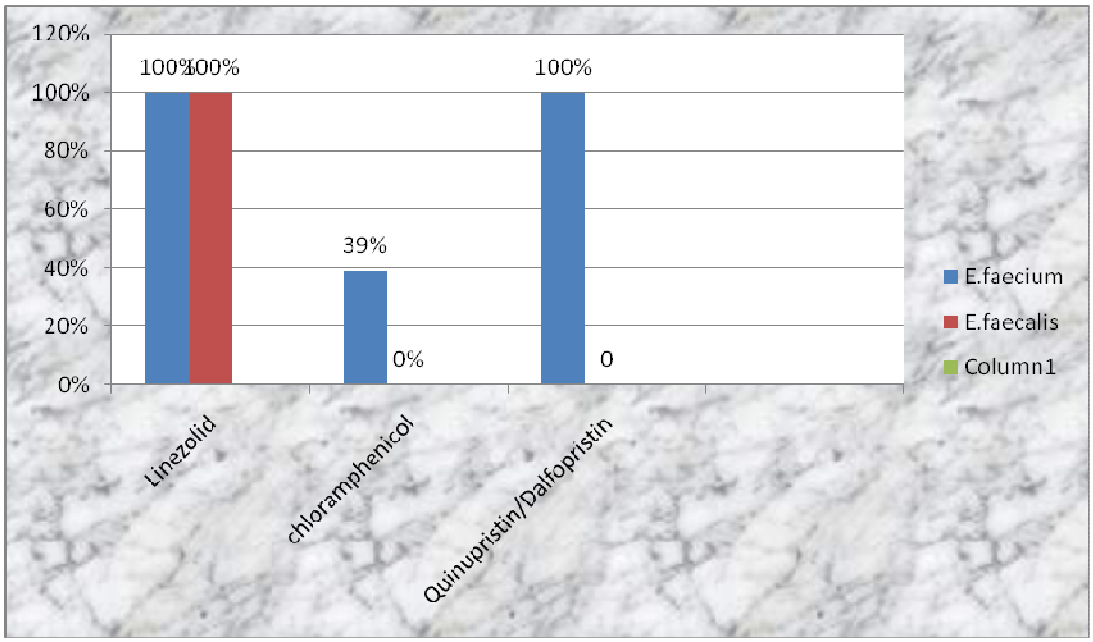


Figure 10.Antimicrobial Susceptibility pattern of VRE isolates

DISCUSSION:

Enterococci are emerging as one of the most common agents of nosocomial infections in the hospital and also cause opportunistic infections in immunocompromised individuals[6,7,11]. It is a well known fact they can cause a variety of serious life threatening infections like endocarditis, bloodstream infections and also cause wound infections , urinary tract infections[6,7,44]. Their survival ability under adverse environmental conditions along with the property of intrinsic and acquired resistance to a variety of antibiotics make them the difficult pathogen to treat with significant mortality and morbidity[7]. Hence it is essential to detect them early and institute proper therapy based on the antimicrobial susceptibility pattern. With the emergence of Vancomycin Resistant Enterococci (VRE), the situation has worsen leaving very few options of selecting antibiotics for the treatment of this multi – drug resistance organism. At this context, our study has been attempted to assess the prevalence of vancomycin resistance among the clinical isolates of Enterococci recovered from the patients in this area.

A total of about 240 *Enterococcal* isolates were recovered from a total of about 21,045 clinical samples including urine , blood ,pus. The majority of the specimens were from inpatients (76%) than from

outpatients(24%) which is in correlation with the findings of Acharya[1] et al,who have reported 72% specimens from hospitalized patients and 28% specimens from outpatients.

In our study, majority of the *Enterococcal* isolates were from urine specimens 211 (87%) , followed by blood (5%),pus (3%) tissue fluids (0.8%),feces(4.6%).which is higher than the findings of V.Gupta. et al[46]. who have reported *Enterococcal* isolation rate of 49% from urine,5% from blood

In this study , a total of 14 (6%) *Enterococci* isolates were from intensive care units (medicine, surgery). Suzanne.L.F et al[41]. have reported 13.9% of *Enterococcal* isolates from ICU and 12% from non ICU patients . In our study, the isolation rate from ICU patients is lower about 6%. The risk factorswe observed in ICU patients were presence of intravenous catheters , one patient on endotracheal intubation,two patients were transplant recipients under immunosuppression,one patient on urinary catheterization, presence of comorbid conditions such as Diabetes and heart disease, prolonged hospitalization and broad spectrum antibiotic usage such as third generation cephalosporins.From nephrology 3 patients were on chronic ambulatory peritoneal dialysis.In the surgical wards, presence of

comorbid conditions like diabetes, prolonged hospitalization , broad spectrum antibiotic usage (third generation cephalosporins, metronidazole)were the associated risk factors. The pus samples from surgical patients were chronic ulcers, and discharge from abdominal wound(no drain). In medicine patients comorbid conditions, broad spectrum antibiotic usage and recurrent infections were the associated risk factors. In pediatric patients, malnutrition and prolonged hospitalization were observed. The feces samples were processed as surveillance cultures.

We also observed that the isolation rate in patients of medicine was 21%,whereas it was 8% from surgery patients and 21% from pediatrics .. The present study showed varying isolation rates with other studies such as MM. Salem-Bekhit et al[39] , who have reported the *Enterococcal i*solation rate of about 85% from ICU, about 27.9% from surgical patients and11.3% from internal medicine ward.

Out of the total 240 *Enterococci* isolated, majority were isolated from adult patients189 (79%),however around 51(21%)of isolates from pediatric patients. Acharya,A. et al[1]. have isolated about 30.5% *Enterococci* from pediatric patients a which is in close resemblance to our study.

A higher isolation rate of about 57% (138/240) was observed among the female patients than 43% (102/240) from male patients. This is in contrary to the findings of MM Salem – Behkit et al[39]. who have reported a male preponderance of about 91% in their study.

In our study we observed that *E.faecalis* is the predominant species 126 (52.5%) followed by *E.faecium* 84 (35%) *E.raffinosus*(3%), *E.durans(0.*8%) E.hirae 0.8% , *E.avium(0.4%) and E,mundtii (0.4%)* .In other studies , Perlada.D,et al[37]. have reported,69%E.faecalis,29% *E.faecium* and 1% each of *E.avium and E.durans* .Vittal P Prakash et al[47] have reported 2.5% *E.raffinosus* and 2.5% *E. hirae* , 1.7% *E.mundtii* .and MM Salem-Behkit et al[39]. have reported 2.1% *E.avium* and 0.8% of *E.hirae* which correlates with the findings of our study.

On studying the antibiotic susceptibility pattern we found that most of *E. faecalis* isolates are highly resistant to penicillins (91%) but a higher sensitivity to ampicillin (87%) .The *E .faecium* isolates have shown higher resistance rates to penicillin(99%), ampicillin (79%) doxycycline(68%)

Agarwal.J[2]. et al, have reported significantly higher resistance to ampicillin among *E.faecium* isolates than *E.faecalis* similar to our study. Multidrug resistance i.e resistance to penicillin,doxycycline,

and aminoglycoside, is a common finding in our study among the *E,faecium* isolates as observed by the studies of Ghoshal.U et al[22]. (2006) from Lucknow, central India , Agarwal.J et al[2]. (2008) from Lucknow.

As per our study,the total HLAR- high level aminoglycoside resistance observed is 73%.The HLAR is observed in *E.faecalis* isolates was 68% and about 80% in *E.faecium.* Our finding is similar to the report by Mohanty.S et al[33] from north India HLAR rate about 73.3% and 77% by Ghoshal.U et al[22] from Lucknow. Also resistance to streptomycin only is observed in a total of70 isolates (33%) and resistance to gentamicin only is observed in42 isolates (20%) in our study which is a little higher than the reports from MM Salem-Behkit et al[39] from Saudi Arabia20.9% to gentamicin and 19.4% for streptomycin.

Out of the240 *Enterococcal* isolates, the29 isolates(12%) which were identified presumptively as vancomyin resistant on vancomycin screen agar containing vancomycin 6µg/ml were subjected to MIC (Minimum Inhibitory Concentration) for vancomycin as a confirmatory test. Of these 29 isolates, 8 isolates turned out to be susceptible by vancomycin MIC (≤4µg/ml) and 21 isolates were vancomycin resistant with 11 isolates showing vancomycin MIC values 8-16µg /ml ,and 10

isolates ≥32µg/ml In our study, the MIC range of most of theVRE isolates fall within 8-256µg/ml with one isolate showing 512µg/ml.This was similar to the findings of Patel.Ret al[52], from mayo clinic, United States, the vancomycin MIC range of VRE isolates 8-256µg/ml in their study. Praharaj.I et al[38]. from South India have reported a case report, van A *E.faecalis* with Vancomycin MIC 256µg/ml,.

All the 21 vancomycin resistant isolates were tested for MIC of teicoplanin and 11 isolates showed The MIC in the susceptible range (0.5-1 µg/ml) and 5 in intermediate range-16 µg/ml and 5 in resistant range(>32 µg/ml).Thus in our study, the teicoplanin MIC range observed was16-64µg/ml which was similar to the report given by Patel.R etal[52]. from mayo clinic with isolates showing>16 µg/ml and by Ghoshal. U et al[22] .>16µg/ml in all their VRE isolates.

Based on the MIC of vancomycin and teicoplanin , 10 VRE isolates (10/21) (48%) were of vanA phenotype showing resistance to both vancomycin(≥64 µg/ml) and teicoplanin(≥16 µg/ml). The remaining11 isolates belong to VanB phenotype (11/21) (52%) with vancomycin MIC ≥8 µg/ml and teicoplanin MIC in the susceptible range usually (0.5-1 µg/ml). Thus a total of about 21(8.7%)of both

E.faecium and *E.faecalis* VRE isolates have been identified in our study. Agarwal.J, et al[2], from have reported a VRE isolation rate of 4.65% in *E.faecalis* in their study . Ghoshal .U et al[22]. from from Lucknow have reported a 1.4% (10/685) VRE isolation (all were *E.faecium)* in their study which is lower than our study.

Out of the total 21 VRE isolates 1 *E.faecalis* and 9 *E.faecium* isolates were of vanA genotype as per the PCR assay for vanA gene . The remaining 11 VRE isolates did not show any band corresponding to vanA gene. Hence They may belong to any of the genotype other than vanA. Yasliani .S et al[51]. have reported in his study 10(58%) vanA genoytypes, 6(35%) VanB genotype(6%) and 1VanC genotype in a total of 17 VRE isolates urine, blood and stool. In our study we have isolated 10(48%) vanA genotype VRE out of 21 VRE isolates which is slightly lower compared to the above study. There are isolated case reports of vanA *E,faecalis* by Praharaj. I.et al[38] from south India. Roger .M.Faucher et [a49l]. from Canada have reported 25% (55/223) vanA *E.faecium* which is little lower rate than our study.

About 9 *E.faecium* isolates and 1 *E,faecalis* shows High level resistance to both Vancomycin and Teicoplanin and are of VanA phenotype. Thus there is 100% concordance of phenotypic classification

by Vancomycin MIC and genotypic detection of the VanA resistance type. Similar to our study, Suzanne et al[41] have reported 100% concordance of these two methods for the detection of van A VRE. Whereas Perlada.D et al[52] have reported 95% concordance in their study which is lower than that of our study.

On studying the susceptibility pattern of supplemental drugs like linezolid, Quinupristin/ dalfopristin and chloramphenicol , all of the he VRE isolates were susceptible to Linezolid(100% sensitivity), 67%showed resistance to chloramphenicol and all of the *E. faecium* VRE isolates were sensitive to Quinupristin/Dalfopristin. The study carried out by V,Gupta,et al[46], from Chandigarh , India and MM Salem- Behkit et al[39]. from Iran have reported, 100% sensitivity of VRE isolates to linezolid which is similar to our study. Perlada.D[37].et al, from Australia also have reported 100% sensitivity to linezolid and 100 % sensitivity to chlramphenicol. But in our study only 39% of isolates were sensitive to chloramphenicol.

SUMMARY:

- A total of about 21,045 clinical specimens like urine , blood, pus and tissue fluids were analyzed for *Enterococcal* isolates.Majority of the specimens were from inpatients (76%) than from outpatients.
- A total of about 240 *Enterococcal* isolates were recovered from these specimens. Majority of the isolates were from urine 87% followed by blood 5%and pus 3% fluids (0.8%).
- A total of about 14 (6%) *Enterococcal* isolates were from intensive care units and the isolation rate from other specialities - nephrology 11(4,5%), urology 92(38%), surgery 20 (8%), medicine 50 (21%) and paediatrics 51 (21%).
- Out of the 240 isolates,majority were from adults about 189(79%) and 51 (21%) from children. Higher isolation rate of about 57% (138/240) was observed in female patients when compared to male patients43% (102/240).
- *E. faecalis* was the predominant *Enterococcal* species with an isolation rate of about 52.5% in our study, followed by *E.faecium 35%.*

- Other than these two species, *E.raffinosus(3%) , E.sulfurous(2%)*, *E.durans(0.8%), E.hirae (0.8%) E.avium (0,4%)and E. mundtii(0.4%)* were also recovered from the clinical specimens.
- The antibiotic susceptibility pattern showed 99% resistance to penicillin,79%to ampicillin, 68% doxycycline resistance in *E.faecium* isolates and 91% penicillin resistance ,13% ampicillin resistance and 64% doxycycline resistance in *E.faecalis* isolates.
- The HLAR- high level aminoglycoside resistance observed is 73% and resistance to both agents observed in a total of 42 isolates (20%) and resistance to streptomycin only is observed in a total of70 isolates (33%) and resistance to gentamicin only is observed in42 isolates (20%).
- The High Level Aminoglycoside Resistance was higher in *E.faecium* isolates 80% (68/84) than *E.faecalis* isolates 86/126(68 %).
- About 29 isolates (12%) were presumptively identified as vancomycin resistant by vancomycin screen agar containing 6 µg/ml vancomycin.
- All the 29 isolates were tested for Minimum Inhibitory Concentration(MIC) of vancomycin by microbroth dilution technique which showed 8 isolates to be vancomycin susceptible

(MIC values ≤4µg/ml) and 21 isolates with vancomycin MIC range of ≥8µg/ml (11 isolates in intermediate range 8-16 µg/ml and 10 isolates in resistant range ≥32µg/ml),

- The highest MIC value observed being 512µg/ml by *E.faecium* isolated from pus specimen from a patient with pseudocyst of pancreas. The other VRE isolates - one *E.faecium* from blood sample of a patient with heart disease with suspected infective endocarditis ,one *E.faecium* from bronchial secretions in a patient with suspected carcinoma bronchus, 1 *E.faecium and 1 E,faecalis* from pus(leg ulcer, ear discharge) and the remaining isolates *2E.faecalis* and14 *E.faecium* from urine specimens from patients with comorbid conditions like diabetes,hypertension and decompensated liver disease.feces samples were negative for VRE.
- The commonly associated risk factors observed in these VRE patients were broadspectrum antibiotic usage especially third generation cephalosporins and metronidazole , longer duration of hospital stay, presence of implants such as endotracheal tube and presence of comorbid conditions like Diabetes, Hypertension and heart disease.

- The MIC testing of Teicoplanin by microbroth dilution technique for these 21 isolates (to assess the Vancomycin Resistance Phenotype- VanA,VanB,VanC, Van D, VanE and VanL) showed -11 isolates were susceptible to teicoplanin (MIC 0.5-1µg /ml) and 10 isolates were resistant ≥ 16µg/ml.
- About 10 VRE isolates showed MIC ≥64µg/ml for Vancomycin and ≥16µg/ml for teicoplanin and are identified as of VanA Phenotype[28,44].
- The remaining 11 isolates showed Teicoplanin MIC in the range of 0.5-1µg/ml and thus belong to VanB Phenotype[28,44].
- Hence the total VRE isolates as per vancomycin MIC values were 21 and thus the prevalence rate was 8.7% (21/240) .
- All the 21 VRE isolates were subjected to detection of Vancomycin Resistance gene - Van A by Polymerase Chain Reaction.
- As per the PCR results, a total of 10 isolates including 9 *E.faecium* and one *E.faecalis* were found to be of VanA Genotype, which were identified as vanA phenotype by MIC of vancomycin and teicoplanin.

- Thus in our study we found 100% concordance between Phenotypic classification by Vancomycin MIC detection and molecular genotyping for the detection of Van A type of VRE.
- All the 21 VRE isolates were sensitive to Linezolid and11 (39%) were sensitive to chloramphenicol. All the 18 vancomycin *E.faecium* were sensitive to Quinupristin and Dalfopristin.

CONCLUSION:

Enterococci are emerging as an important pathogen causing variety of hospital acquired nosocomial infections and also cause community acquired infections contributing significantly to patients morbidity and mortality. The emergence of vancomycin resistant *Enterococci* worsens the problem further because of the multidrug resistance exhibited by these agents leaving fewer therapeutic options for the clinicians in treating the serious life threatening VRE infections. In our study we isolated a total of 240 Enterococcal isolates from various clinical samples with *Enterococcus faecalis and Enterococcus faecium* as the predominant species . Of these 21 isolates were identified as Vancomycin Resistant Enterococci with a prevalence rate of about 8.7% as per Vancomycin MIC and the prevalence of VanA genotype VRE is 4.1% by PCR assay in this region. They showed resistance to multiple antibiotics like penicillin, ampicillin, doxycycline and exhibited higher rate of high level aminoglycoside resistance. The *Enteroccocal* isolates presumptively identified by vancomycin screen agar should be confirmed by determining the minimum inhibitory concentration for vancomycin. The phenotyping of VRE isolates performed by detection of MIC for both vancomycin and teicoplanin

correlates well with the genotypic method of detection of vancomycin resitance geneVanA. Thus this method can be adopted in resource limited settings (where the genotyping may not be available) for the detection of Vancomycin resistant phenotype of *Enterococci.*

This emphasizes the need for conducting frequent surveillance programmes for prompt identification of VRE in hospitals and community. This also emphasizes the need for implementation of stringent infection control measures like rational use of antibiotics especially restricting the use of Vancomycin to minimum, proper containment and effective treatment of VRE infections, strict hand washing practices, education of the healthcare workers and other personnel involved in the patient management. These measures are to be strictly followed to bring down the mortality and morbidity associated with these nosocomial VRE infections.

APPENDIX

Blood agar:

Ingredients

Sterile sheep blood -5 ml

Nutrient agar -100 ml

Autoclave the nutrient agar base at 121º C for 15 minutes. Cool to 45-50º C and add blood with sterile precautions and pour into Petri dish plates.

MacConkey Agar

Ingredients		Grams/litre
Peptic digest of animal tissue	-	17
Proteose peptone	-	3
Lactose	-	10
Bile salts	-	1.5
Sodium chloride	-	5
Neutral red	-	0.03
Agar	-	15

Final pH at (25º C) 7.1±0.2.

Suspend 51.53 grams in 1000 ml of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. Mix well and pour into petri dish plates.

Mueller Hinton Agar:

Ingredients

Beef infusion - 300 g/l

Casein acid hydrolysate - 17.50 g/l

Starch -1.50 g/l

Agar -17.00 g/l

Final pH at 25º C 7.4.

Suspend 38 gms in 1000 ml of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. Mix well and pour 20-25 ml of it into petri dishes of 90 mm diameter to a depth of 4 mm of medium.

Bile Esculin Agar:

Ingredients :

Peptone - 5 gm

Beef extract -3gm

Oxgall(bile) -40gm

Esculin -1gm

Ferric citrate -0.5gm

Agar -15gm

Distilled water -1 L

pH 7.0

heat to dissolve the contents completely, sterilize at autoclave at 121ºC for10 minutes, pour into slants/ petri plates.

6.5% NaCl broth:

Nutrient broth - 1L

NaCl - 6.5gm

Dissolve the contents completely , autoclave at 121ºc for15 min and distribute in tubes.

Brain -Heart infusion agar:

Ingredients :

Agar - 15gm

Bran heart infusion broth -1L

pH 7.4

Dissolve the agar completely by boiling . autoclave at 121ºc for 15 min. cool to about 50ºC and pour into petri dish plates.

Vancomycin Screen agar:

Ingredients :

Agar - 15gm

Brain heart infusion broth - 1 L

Vancomycin - 6mg/L

Prepare Brain heart infusion agar as described above , cool to 50ºC and add

Vancomycin 6µg/ml , mix well and pour into petri dish plates.

Cation Adjusted Mueller –Hinton broth: (MHA broth 2) (Himedia lab).

Cation adjusted Mueller- Hinton broth base - 21 gm

Distilled water -1L

Dissolve the contents by boiling and sterilize by autoclaving at 121ºC for 15 min.

TABLE. 1.ZONE DIAMETER INTERPETIVE STANDARDS FOR *ENTEROCOCCUS SPP.*

Antimicrobial agent	Disk content	Zone diameter		
		R	IM	R
Penicillin G	10units	≤14	-	≥15
Ampicillin	10µg	≤16	-	≥17
Erythromycin	15µg	≤13	14-22	≥23
Ciprofloxacin	5µg	≤16	16-20	≥21
Doxycycline	30µg	≤12	13-15	≥16
HLS – high level gentamicin	120µg	6	7-9	≥10
HLG- high level streptomycin	300µg	6	7-9	≥10

Vancomycin	30µg	≤14	15-16	≥17
Teicoplanin	30µg	≤10	11-13	≥14
Chloramphenicol	30µg	≤12	-	≥18
Linezolid	30µg	≤20	-	≥23
Quinupristin/ dalfopritin	15µg	≤15	16-18	≥19

R-resistant, IM-intermediate S – sensitive

Table 2 MIC Interpretive standards 9µg/ml for *Enterococcus spp.*

ANTIMICROBIAL AGENT	MICµg/ml INTERPRETIVE STANDARD		
	S	I	R
Vancomycin	≤4	8-16	≥32
Teicoplanin	≤8	16	≥32

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KEY TO MASTER CHART:

IP-Inpatient

M-Male patient

F-Female patient

Mch-Male child

Fch-Female child

UTI- Urinary tract infection

PUO- Pyrexia of unknown origin

FFE –Fever for evaluation

CKD- Chonic kidney disease

CKD-Tx –Renal transplant recipient

CA- BR-Carcinoma bronchus

Pseudo-cys – Pseudocyst of pancreas

S- Susceptible R-Resistant

vanA – van A genotype VRE

PRH- prolonged hospitalization

Abuse – broad spectrum antibiotic use

ET- tube- Endotracheal tube

S.NO	AGE/SEX	WARD	RISK FACTORS	SAMPLE	ISOLATE	ANTIBIOTIC SUSCEPTIBILITY	VRE PHENOTYPE	VRE GENOTYPE
A2	47/F	S-IP	PH,AB use	pus	E.faecalis	R-all	vanB	
A4	39/F	U-OP	R-UTI,AB use	urine	E.faecalis	R-all	vanA	vanA
A21	8/12Mch	P-IP	AGE	stool	E.faecium	S-Gentamycin	vanA	vanA
A23	55/M	M-OP	R-UTI, PRH,AB use	urine	E.faecium	R-all	vanB	
A29	55/M	M-OP	R-UTI, fever	urine	E.faecium	S-Gentamycin	vanB	
B3	2/Mch	ISP-IP	AB use, HP	urine	E.faecium	S-Streptomycin	vanA	vanA
B4	36/F	U-OP	Fever, AB use	urine	E.faecium	S-Streptomycin	vanA	vanA
B6	38/M	M-OP	PRH	urine	E.faecium	R-all	vanB	
B7	55/F	U-OP	AB use,DM	urine	E.faecium	R-all	vanA	vanA
B64	72/F	M-OP	DM, R-UTI, HP	urine	E.faecium	R-all	vanA	vanA
C27	45/M	MGE-IP	HP,AB use	urine	E.faecium	S-Doxycycline	vanA	vanA
C52	8/Fch	P-IP	R-UTI,HP	urine	E.faecium	S-Ampicillin	vanA	vanA
C61	32/M	SGE-IP	HP,AB use	pus	E.faecium	S-Doxycycline and streptomycin	vanA	vanA
C92	28/F	ENT-IP	HP,AB use	pus	E.faecium	S-Streptomycin	vanB	
UN1	58/M	IMCU-IP	DM, AB use, HP	urine	E.faecium	S-Ampicillin,Doxycycline	vanA	vanA
UN7	55/M	U-OP	DM,R-UTI	urine	E.faecalis	S-Streptomycin, Ampicilllin	vanB	
UN8	30/F	RSRM-IP	HP,AB use	urine	E.faecium	S-Streptomycin, Ampicilllin	vanB	
4	42/M	CM-IP	ET tube, HP, AB use	bronch. Secrt	E.faecium	S-Gentamycin	vanB	
9	52/M	N-IP	HMD,HP,AB use	urine	E.faecium	S-Gentamycin	vanB	
24	60/F	ICU-IP	DM,R-UTI,HP,AB use	urine	E.faecium	S-Ampicillin,Doxycycline	vanB	

S.no	Age	Sex	OP/IP	Clinical diagnosis	Specimen	Isolate	Antibiotic susceptibility											
							Penicillin	Ampicillin	Erythromycin	Ciprofloxacin	Doxycycline	HLS	HLG	Teicoplanin	Vancomycin	VRE screen agar	MIC-Vancomycin	VRE-Genotype
A2	47	F	IP	ulcer-leg	Pus	*E.faecalis*	R	R	R		R	R	R	R	R	POS	R	vanA
A4	39	F	OP	UTI	Urine	*E.faecalis*	R	R		R	R	R	R	R	R	POS	R	vanA
A5	55	F	IP		Bile	*E.faecium*	R	R	R		R	S	S	S	S	Neg		
A6	2	Fch	IP	PUO	Urine	*E.faecalis*	R	S			S	S	R	S	S	Neg		
A7	65	M	IP	PUO	Urine	*E.faecium*	R	R		R	S	R	R	S	S	Neg		
A8	37	M	IP	CKD	Urine	*E.faecalis*	R	S		S	S	S	R	S	S	Neg		
A13	7	Fch	IP	PUO	Urine	*E.faecium*	R	R			S	R	R	S	S	Neg		
A14	60	M	IP	UTI	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
A18	33	F	IP	UTI	Urine	*E.faecium*	R	S		S	S	S	S	S	S	Neg		
A21	8M	Mch	IP	AGE	Feces	*E.faecium*	R	R			R	R	S	R	R	POS	R	vanA
A22	21	M	IP	PUO	Urine	*E.faecium*	R	R		R	R	R	R	S	S	Neg		
A23	55	M	OP	UTI	Urine	*E.faecium*	R	R		R	R	R	R	R	R	POS	R	vanA
A25	28	F	OP	UTI	Urine	*E.faecalis*	R	S		S	R	S	S	S	S	Neg		
A27	6	Mch	IP	PUO	Blood	*E.dispar*	R	R	R		R	S	S	S	S	Neg		
A28	19	F	OP	CKD	Urine	*E.faecium*	R	R		R	R	S	R	S	S	Neg		
A29	55	M	OP	UTI	Urine	*E.faecium*	R	R		R	R	R	S	R	R	POS	R	vanA
A30	79	M	IP	UTI	Urine	*E.faecalis*	R	S		R	R	R	R	S	S	Neg		
A31	11M	Mch	OP	PUO	Urine	*E.faecalis*	R	S		R	R	S	R	S	S	Neg		
A33	35	F	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
A34	20	F	IP	PUO	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
A35	6	Fch	OP	PUO	Urine	*E.faecalis*	R	S		R	S	S	S	S	S	Neg		
A36	35	F	IP	UTI	Urine	*E.faecalis*	R	R		R	R	R	R	S	S	Neg		
A37	4	Fch	IP	PUO	Urine	*E.faecalis*	R	R		R	R	R	R	S	S	Neg		
A68	11	Fch	IP	PUO	Urine	*E.faecium*	R	R		R	S	R	R	S	S	Neg		
A69	80	F	IP	UTI	Urine	*E.faecium*	R	R		R	R	R	S	S	S	Neg		
A70	32	M	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	R	S	S	Neg		
A71	40	F	IP	UTI	Urine	*E.faecium*	R	R		R	R	S	R	S	S	Neg		
A72	8	Fch	IP	PUO	Blood	*E.faecalis*	R	S	R		R	R	S	S	S	Neg		
A73	47	M	IP	PUO	Blood	*E.faecalis*	R	S	R		R	R	S	S	S	Neg		
A83	30	M	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
A88	21	M	OP	UTI	Urine	*E.faecium*	R	R		R	R	R	R	R	S	POS	S	
A91	28	F	IP	UTI	Urine	*E.faecalis*	R	S		R	S	R	S	S	S	Neg		
A92	30	M	IP	UTI	Urine	*E.faecalis*	S	S		R	R	S	R	S	S	Neg		
A97	4	Mch	IP	PUO	Urine	*E.faecalis*	S	S		R	R	S	S	S	S	Neg		
3	27	F	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
4	42	M	IP	CA-BR	BR-SEC	*E.faecium*	R	R	R	R		R	S	R	R	POS	R	vanA

8	62	F	IP	PUO	Urine	*E.faecalis*	R	S		R	R	R	R	S	S	Neg		
9	52	M	OP	CKD	Urine	*E.faecium*	R	R		R	R	R	S	R	R	POS	R	vanA
10	39	F	OP	UTI	Urine	*E.faecium*	R	S		R	S	S	S	S	S	Neg		
11	12	Mch	OP	UTI	Urine	*E.faecalis*	R	S		R	R	R	R	S	S	Neg		
13	22	Fch	OP	UTI	Urine	*E.faecium*	R	R		R	R	S	R	S	S	Neg		
22	13	F	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
24	60	F	IP	DM	Urine	*E.faecium*	R	S		S	S	S	S	R	R	POS	R	vanA
25	15	F	OP	UTI	Urine	*E.feacium*	R	R		R	S	R	R	S	S	Neg		
40	62	F	IP	LRI	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
43	32	M	OP	PUO	Urine	*E.faecium*	R	R		R	S	S	R	S	S	Neg		
44	31	F	IP	UTI	Urine	*E.faecalis*	R	S		R	R	R	R	S	S	Neg		
45	43	M	IP	DM-HD	Feces	*E.faecium*	R	R		R	R	S	R	S	S	Neg		
46	35	M	IP	HD-DM	Feces	*E.faecium*	R	S		R	R	S	S	S	S	Neg		
47	38	F	OP	PUO	Urine	*E.faecalis*	R	R		R	R	S	R	S	S	Neg		
48	32	M	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
50	40	F	OP	PUO	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
51	49	F	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
54	55	M	OP	PUO	Urine	*E.faecalis*	R	S		R	S	S	S	S	S	Neg		
55	58	F	OP		Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
57	37	F	OP	LRI	Urine	*E.columbae*	R	S		S	S	S	S	R	R	POS		
58	9	Fch	OP	UTI	Urine	*E.faecium*	R	R		R	R	R	S	S	S	Neg		
60	22	F	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
61	65	F	IP	DM	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
X1	65	F	IP	DM-HT	Urine	*E.faecalis*	R	R		R	R	S	R	S	S	Neg		
X2	24	F	IP	PUO	Urine	*E.faecalis*	R	S		R	R	R	S	S	S	Neg		
X3	6M	Mch	IP	PUO	Blood	*E.faecalis*	R	S		R			S	S	S	Neg		
X4	17	F	OP	PUO	Urine	*E.faecium*	R	R		R	S	S	S	S	S	Neg		
X5	23	F	IP	DLD	Blood	*E.faecium*	R	S	R	R	S	S	R	S	S	Neg		
X7	37	F	OP	UTI	Urine	*E.faecium*	R	R		R	S	S	R	S	S	Neg		
X8	27	F	IP	CKD	Urine	*E.cCPC PNSE*	R	S		R	R	S	R	R	R	Neg		
X9	10	Fch	IP	PUO	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
X10	55	M	IP	CKD-TX	Urine	*E.faecalis*	R	R		R	S	R	R	S	S	Neg		
X12	60	F	OP	VD	Urine	*E.faecalis*	R	R		S	R	S	R	S	S	Neg		
S3	7	Mch	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
S6	18	F	IP	PUO	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
F1	69	F	OP		Urine	*E.faecium*	R	S		R	R	S	R	S	S	Neg		
F3	8M	Mch	IP	PUO	Urine	*E.faecalis*	R	S			S	S	S	S	S	Neg		
UN1	58	M	IP		Urine	*E.faecium*	R	S		R	S	S	S	S	S	Neg		
UN2	55	F	IP	DM-HD	Urine	*E.faecalis*	R	S		R	R	S	R	R	R	POS	R	
UN3	40	F	OP		Urine	*E.faecium*	R	S		R	R	S	R	R	R	POS		
UN5	31	M	IP		Feces	*E.faecalis*	R	S		R	R	S	R	S	S	Neg		
UN7	55	M	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	R	R	R	POS	R	vanA
UN8	30	F	IP	POST-OP LS(	Urine	*E.faecium*	R	S		R	R	S	R	R	R	POS	S	

S7	13	F	OP	PUO	Blood	*E.faecalis*	R	S	R		R	R	R	S	S	Neg		
S8	37	F	OP		Urine	*E.faecalis*	R	S		R	S	S	S	S	S	Neg		
S9	63	F	IP	DM-HD	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
S10	15	F	OP	UTI	Urine	*E.faecalis*	S	S		R	R	S	S	S	S	Neg		
S11	63	M	IP	DM-LRI	Urine	*E.faecalis*	S	S		R	R	S	S	S	S	Neg		
B1	43	F	IP	DM-SEP	Blood	*E.feacium*	R	R	R	R	R	R	S	R	R	POS	R	vanA
B2	11m	Mch	OP	PUO	Urine	*E.faecalis*	R	S				S	S	S	S	Neg		
B3	2	Mch	IP	PUO	Urine	*E.faecium*	R	R		R	S	S	R	R	R	POS		
B4	36	F	OP	UTI	Urine	*E.faecium*	R	R		R	R	S	R	R	R	POS	R	vanA
B5	75	M	IP	POP-turp	Urine	*E.faecium*	R	R		R	R	S	R	R	R	POS		
B6	38	M	OP	UTI	Urine	*E.faecium*	R	R		R	R	R	R	R	R	POS	R	vanA
B7	55	F	OP	UTI	Urine	*E.faecium*	R	R		R	R	R	R	R	R	POS		
B8	4	Fch	IP	PUO	Urine	*E.faecalis*	R	R		R	R	S	R	S	S	Neg		
B9	15	M	OP	PUO	Urine	*E.faecalis*	R	S		R	R	R	R	S	S	Neg		
B11	45	F	OP	PUO	Urine	*E.faecalis*	R	S		R	S	S	R	S	S	Neg		
B12	65	M	IP	jaundice	Urine	*E.faecalis*	R	R		R	R	S	R	S	S	Neg		
B13	2	F	IP	PUO	Urine	*E.faecium*	R	S		S	S	S	S	S	S	Neg		
B14	4	F	IP	PUO	Urine	*E.faecalis*	R	S				S	S	S	S	Neg		
B15	47	F	IP	DM	Urine	*E.faecium*	R	R		R	R	S	R	S	S	Neg		
B17	49	F	IP	acute abd	Urine	*E.faecalis*	R	S		R	S	S	S	S	S	Neg		
B18	6	Mch	OP		Urine	*E.faecalis*	R	S			R	R	S	S	S	Neg		
B19	58	F	OP	UTI	Urine	*E.faecium*	R	R		R	R	S	R	S	S	Neg		
B20	10	M	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	R	S	S	Neg		
B21	2	Mch	IP	PUO	Blood	*E.columbae*	R	S	R			S	S	S	S	Neg		
B22	55	F	IP	UTI	Urine	*E.faecium*	R	R		S	R	S	R	S	S	Neg		
B23	22	M	IP	PUO	Urine	*E.faecalis*	R	S		S	S	S	S	S	S	Neg		
B24	48	F	IP	UTI	Urine	*E.faecalis*	S	S		R	S	S	S	S	S	Neg		
B25	40	F	IP	PUO	Urine	*E.faecalis*	R	R	R	S	S	S	S	S	S	Neg		
B26	8	Mch	IP	PUO	Urine	*E.faecium*	R	S			S	S	R	S	S	Neg		
B27	8	Mch	OP		Urine	*E.faecalis*	R	S			S	S	S	S	S	Neg		
B28	20	F	IP	MNG	Urine	*E.faecalis*	R	S		S	S	S	S	S	S	Neg		
B32	30	F	IP	UTI	Urine	*E.faecalis*	R	R		S	S	R	S	S	S	Neg		
B53	55	M	OP	UTI	Urine	*E.faecalis*	R	R		R	R	S	R	S	S	Neg		
B54	19	F	OP		Urine	*E.faecium*	R	R		R	R	S	R	S	S	Neg		
B55	7	M	OP	PUO	Urine	*E.faecalis*	R	S				S	S	S	S	Neg		
B57	6M	Mch	IP	PUO	Urine	*E.faecalis*	R	S			R	R	R	S	S	Neg		
B60	52	M	IP	HD-DM	Urine	*E.faecium*	R	R		R	S	R	R	S	S	Neg		
B61	12	F	IP	PUO	Urine	*E.faecalis*	R	S		R	S	S	S	S	S	Neg		
B64	72	F	IP	UTI	Urine	*E.faecium*	R	R		R	R	R	R	R	R	POS	R	vanA
B65	46	F	OP	UTI	Urine	*E.faecium*	R	R	R	R	S	R	R	S	S	Neg		
B67	30	F	OP	PID	Urine	*E.raffinosus*	R	R		S	S	R	S	S	S	Neg		
B68	9	M	IP	PUO	Urine	*E.raffinosus*	R	S				S	R	S	S	Neg		
B69	30	M	OP		Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		

B70	25	M	OP	UTI	Urine	*E.faecalis*	R	S		S	S	S	S	S	S	Neg		
B71	31	F	IP	CKD	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
B72	47	M	IP	HD-IE	Blood	*E.faecalis*	R	S	R		R	S	S	S	S	Neg		
B74	30	M	OP	UTI	Urine	*E.faecium*	R	S		R	S	S	R	S	S	Neg		
B75	8	Fch	OP		Urine	*E.faecalis*	R	R				R	R	S	S	Neg		
B76	21	M	IP	PID	Urine	*E.faecium*	R	S		R	R	S	R	S	S	Neg		
B77	51	M	IP	ASCITIS	Urine	*E.faecalis*	R	R		R	R	S	R	S	S	Neg		
B80	1	Mch	IP	PUO	Urine	*E.faecalis*	R	S				S	S	S	S	Neg		
B90	50	F	OP	UTI	Urine	*E.sulfurous*	R	S		S	S	S	S	S	S	Neg		
B92	12	F	OP	PUO	Urine	*E.faecalis*	R	S			S	S	R	S	S	Neg		
B96	30	F	OP	UTI	Urine	*E.faecalis*	R	R		R	S	S	R	S	S	Neg		
B97	64	F	OP		Urine	*E.faecalis*	R	R		R	R	S	S	S	S	Neg		
B98	50	F	OP		Urine	*E.faecalis*	R	R		S	S	R	S	S	S	Neg		
B99	30	M	IP		Urine	*E.faecium*	R	S		S	R	S	R	S	S	Neg		
C3	47	M	OP	PUO	Urine	*E.faecium*	R	R		R	R	R	S	S	S	Neg		
C5	43	F	OP	PID	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
C6	53	M	IP	HD	Urine	*E.faecalis*	R	R		R	R	R	R	S	S	Neg		
C7	24	M	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	R	S	S	Neg		
C8	28	M	IP	HD	Urine	*E.faecalis*	R	S		R	S	S	S	S	S	Neg		
C9	34	M	IP	HYD	Urine	*E.faecium*	R	R		R	R	R	R	S	S	Neg		
C10	43	M	IP	DM- ULCER	Pus	*E.faecium*	R	R	R		R	R	R	S	S	Neg		
C12	32	M	IP	HD	Blood	*E.faecalis*	S	R	R		S	S	S	S	S	Neg		
C14	45	F	OP		Urine	*E.faecalis*	R	R		R	S	R	S	S	S	Neg		
C15	56	M	OP	UTI	Urine	*E.faecalis*	R	R		S	S	R	S	S	S	Neg		
C17	32	F	OP	UTI	Urine	*E.faecalis*	R	R		R	R	R	R	S	S	Neg		
C18	12	Fch	OP	PUO	Urine	*E.faecalis*	R	S		R	S	S	S	S	S	Neg		
C19	6	Mch	IP	PUO	Urine	*E.faecium*	R	R		R	R	R	R	S	S	Neg		
C20	13	F	OP	UTI	Urine	*E.faecalis*	R	S		R	S	S	S	S	S	Neg		
C21	18	F	OP		Urine	*E.faecium*	R	R		R	R	S	R	S	S	Neg		
C23	36	M	IP	ABSECC LLE(	Pus	*E.avium*	R	R	R		S	S	R	S	S	Neg		
C24	43	F	OP	UTI	Urine	*E.faecium*	R	R		R	R	R	R	S	S	Neg		
C27	45	M	IP	LD	Urine	*E.faecium*	R	R		R	S	R	R	R	R	POS		
C30	34	F	IP	LRI	Urine	*E.raffinosus*	R	R		R	S	R	S	S	S	Neg		
C34	21	M	OP	UTI	Urine	*E.faecalis*	R	R		R	S	R	S	S	S	Neg		
C35	34	F	IP	UTI	Urine	*E.faecalis*	R	R		R	S	R	R	S	S	Neg		
C37	72	M	OP	PUO	Urine	*E.faecium*	R	R		R	R	R	R	S	S	Neg		
C38	44	F	IP	HD	Urine	*E.faecalis*	S	S		R	S	R	R	S	S	Neg		
C39	54	M	OP		Urine	*E.faecium*	R	S		R	S	R	S	S	S	Neg		
C40	31	M	IP	Ulcer thigh	Pus	*E.faecalis*	R	S	S		S	S	S	S	S	Neg		
C41	22	M	OP		Urine	*E.durans*	S	S		S	S	S	S	S	S	Neg		
C42	22	M	OP	UTI	Urine	*E.faecalis*	R	R		R	S	R	R	S	S	Neg		
C43	2	McH	OP	PUO	Urine	*E.faecium*	R	R		R	R	R	R	S	S	Neg		
C44	50	F	IP	FEVER	Urine	*E.dispar*	R	S		R	S	R	R	S	S	Neg		

C45	49	F	OP	UTI	Urine	*E.faecium*	R	R		R	R	S	S	S	S	Neg		
C46	53	F	IP		Urine	*E.faecalis*	R	S		R	S	S	R	S	S	Neg		
C47	38	F	OP	UTI	Urine	*E.faecalis*	R	S		R	S	R	R	S	S	Neg		
C48	63	M	OP	CKD	Urine	*E.faecium*	R	R		R	S	R	R	S	S	Neg		
C49	37	F	OP	CKD	Urine	*E.faecium*	R	R		R	R	R	R	S	S	Neg		
C50	8M	Fch	IP	PUO	Urine	*E.faecium*	R	R		R	R	S	R	S	S	Neg		
C51	17	M	IP		Urine	*E.raffinosus*	R	R		S	S	R	S	S	S	Neg		
C52	8	Fch	OP	UTI	Urine	*E.faecium*	R	S			S	S	R	R	R	POS		
C53	35	F	OP	UTI	Urine	*E.faecalis*	R	S		R	R	S	R	S	S	Neg		
C54	60	F	IP	UTI	Urine	*E.faecium*	R	R		S	R	R	R	S	S	Neg		
C55	43	F	OP	UTI	Urine	*E.columbae*	R	R		R	S	R	R	S	S	Neg		
C56	38	M	IP	HD	Pus	*E.faecium*	R	R	R		R	S	R	S	S	Neg		
C57	25	F	OP	UTI	Urine	*E.hirae*	R	S			S	S	R	S	S	Neg		
C58	11	McH	OP	PUO	Urine	*E.faecalis*	R	S			S	S	R	S	S	Neg		
C59	38	M	IP	HD/DM	Urine	*E.faecium*	R	R		R	S	R	R	S	S	Neg		
C60	3	MCh	IP	PUO	Urine	*E.faecalis*	R	S		R	S	S	R	S	S	Neg		
C61	32	M	IP	PSEUDOCYS	Pus	*E.faecium*	R	R	R		S	S	R	R	R	POS	R	vanA
C62	36	F	OP	UTI	Urine	*E.raffinosus*	R	S		S	S	S	S	S	S	Neg		
C63	67	F	IP	CVA	Urine	*E.columbae*	S	S		S	S	S	S	S	S	Neg		
C64	43	F	OP	UTI	Urine	*E.faecalis*	R	S		R	S	S	R	S	S	Neg		
C65	10	Mch	IP	PUO	Urine	*E.faecium*	R	R		R	R	S	S	S	S	Neg		
C66	2	Fch	OP	UTI	Urine	*E.hirae*	S	S		S	R	S	S	S	S	Neg		
C67	21	F	IP	HD	Urine	*E.faecalis*	R	S		R	S	S	S	S	S	Neg		
C68	28	F	OP	UTI	Urine	*E.faecium*	R	R		R	S	S	R	S	S	Neg		
C69	3	Fch	OP		Urine	*E.faecalis*	S	S		S	S	S	S	S	S	Neg		
C70	32	M	IP	Abd-wnd	Pus	*E.mundtii*	S	S	R	R	S	S	S	S	S	Neg		
C71	26	M	IP	Renal Tx	Blood	*E.faecium*	R	R	R		S	S	S	S	S	Neg		
C72	67	F	OP	UTI	Urine	*E.faecalis*	S	S		R	S	R	S	S	S	Neg		
C73	60	F	OP	CKD	Urine	*E.faecalis*	S	S		R	S	S	S	S	S	Neg		
C74	28	F	IP	LSCS -pod	Urine	*E.faecalis*	S	S		S	S	S	S	S	S	Neg		
C75	7	Fch	IP	PUO	Urine	*E.faecium*	R	R			S	R	S	S	S	Neg		
C76	4	Mch	OP	LRI	Urine	*E.faecium*	R	R			R	S	R	S	S	Neg		
C77	30	F	OP	CKD	Urine	*E.sulfurous*	S	S			S	S	S	S	S	Neg		
C78	4	Fch	OP	Fever	Urine	*E.sulfurous*	R	R			R	S	S	S	S	Neg		
C79	3	Fch	OP	Fever	Urine	*E.faecium*	R	S			S	S	R	S	S	Neg		
C80	36	M	OP	UTI	Urine	*E.faecium*	R	S		R	R	S	S	S	S	Neg		
C81	51	M	IP	PUD	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
C82	16	M	OP	UTI	Urine	*E.faecium*	R	R		R	R	S	R	S	S	Neg		
C83	33	M	OP	UTI	Urine	*E.faecalis*	R	S		S	S	S	S	S	S	Neg		
C84	84	F	IP	CVA	Urine	*E.faecium*	R	R		R	S	R	R	S	S	Neg		
C85	37	M	IP	CH-Ulcer leg	Pus	*E.durans*	R	S	R		S	R	R	S	S	Neg		
C86	45	F	OP	UTI	Urine	*E.faecium*	S	S			S	S	S	S	S	Neg		
C87	10	Fch	IP	RHD	Urine	*E.faecalis*	R	S			S	R	R	S	S	Neg		

C88	35	F	OP	UTI	Urine	*E.faecalis*	R	S		S	S	S	S	S	S	Neg		
C89	73	M	IP	PUD-Fever	Urine	*E.faecium*	R	R		R	R	R	R	S	S	Neg		
C90	6	Mch	OP	Fever	Urine	*E.raffinosus*	R	R			R	R	S	S	S	Neg		
C91	18	M	IP	Epilepsy	Urine	*E.faecalis*	R	R			S	R	S	S	S	Neg		
C92	28	F	IP	Ear dis	Pus	*E.faecium*	R	R	R		R	S	R	R	R	POS	R	vanA
C93	25	M	OP	UTI	Urine	*E.faecium*	R	R		R	R	R	R	S	S	Neg		
C94	21	F	OP	UTI	Urine	*E.faecium*	R	S		S	S	S	S	S	S	Neg		
C95	4	Fch	OP	FEVER	Urine	*E.sulfurous*	S	S			S	S	S	S	S	Neg		
C96	20	F	IP	DLD	Urine	*E.faecium*	R	R			R	S	R	S	S	Neg		
C97	8	Mch	OP	FEVER	Urine	*E.faecium*	R	R			S	S	S	S	S	Neg		
C98	37	F	OP	UTI	Urine	*E.sulfurous*	R	R		S	S	R	S	S	S	Neg		
C99	4Day	Fch	IP	Sepsis	Blood	*E.faecium*	R	S	R			S	S	S	S	Neg		
C100	40	F	IP	AGE	Feces	*E.faecalis*	R	S		R	S	R	R	S	S	Neg		
C101	27	F	IP	AGE	Feces	*E.faecalis*	R	S		R	S	S	R	S	S	Neg		
D1	43	M	IP	CH-Ulcer leg	Pus	*E.asini*	R	S	R		S	S	S	S	S	Neg		
D2	32	M	OP	FEVER	Urine	*E.CDC PNS E*	S	S		R	S	R	R	S	S	Neg		
D3	14	M	OP	UTI	Urine	*E.faecalis*	R	S		S	S	S	S	S	S	Neg		
D4	36	F	OP	PID	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
D5	26	F	OP	UTI	Urine	*E.CDC PNS E*	R	S		S	S	S	S	S	S	Neg		
D6	38	M	OP	CKD	Urine	*E.sulfurous*	R	S		R	S	S	R	S	S	Neg		
D7	34	M	IP	HD	Blood	*E.faecium*	R	S	R		R	S	R	R	R	POS		
D8	27	F	OP	FEVER	Urine	*E.raffinosus*	R	S		S	S	S	S	S	S	Neg		
D9	54	M	IP	PUD	Urine	*E.raffinosus*	R	S		R	S	S	S	S	S	Neg		
D10	67	M	OP	DM	Urine	*E.faecalis*	R	S		R	S	S	S	S	S	Neg		
D11	7	Mch	OP	PUO	Urine	*E.asini*	R	S				S	S	S	S	Neg		
D12	47	F	OP	UTI	Urine	*E.faecium*	R	R		R	R	R	R	S	S	Neg		
D13	37	M	IP	CKD	Urine	*E.raffinosus*	R	R		S	S	R	S	S	S	Neg		
D14	49	F	OP	PUO	Urine	*E.faecalis*	R	S		R	R	S	S	S	S	Neg		
D16	9	Mch	OP		Urine	*E.faecium*	R	R				S	R	S	S	Neg		
D17	43	F	OP	UTI	Urine	*E.faecium*	R	S		R	R	R	S	S	S	Neg		
DI8	32	M	IP	CKD	Urine	*E.durans*	S	S		S	S	S	S	S	S	Neg		
D21	28	M	IP	DM	Urine	*E.faecium*	R	S		R	R	S	R	S	S	Neg		

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